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NEUROTOXIN BINDING SITE ON THE ACETYLCHOLINE RECEPTOR

ANNUAL REPORT

Thomas L. Lentz, Maria Gastka, and Diana Donnelly-Roberts

March 1987

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

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Yale University School of Medicine
New Haven, Connecticut 06510

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<p>This research was designed to (1) develop binding-inhibition assays that could be used to screen compounds that might be effective in inhibiting neurotoxin binding to the acetylcholine receptor (AChR), (2) carry out studies to identify the anionic site on primary sequence of the α-subunit to which neurotoxins bind, and (3) determine if antibodies to synthetic receptor peptides might be effective in inhibiting neurotoxin binding to the receptor.</p> <p>A solid phase radioassay was developed to measure the binding of neurotoxins to receptors using binding of 125I-labeled α-bungarotoxin (BTX) to purified Torpedo AChR and membrane-bound AChR as a model. The solid phase assay is rapid, convenient, and utilizes small quantities of reagents. Association and dissociation rate constants and dissociation constants for BTX binding to the AChR determined from kinetic and equilibrium binding data ($K_D = 10^{-11}$) were comparable to the constants observed with</p>					
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in vitro assays (filter disk and centrifugation assays) and to those reported in the literature, thus validating the solid phase assay. Besides α -bungarotoxin, a long, postsynaptic neurotoxin, binding of cobratoxin from Naja naja atra, a short, postsynaptic neurotoxin, and apamin, a centrally acting neurotoxin, could be demonstrated using the solid phase assay. The binding of apamin to brain synaptosomes indicates the assay is sensitive enough to be utilized with preparations containing a low density of receptors. The assay was also employed as a binding-inhibition assay by incubating constant amounts of toxin with increasing concentrations of cholinergic ligands (agonists and antagonists). This assay should be useful in screening compounds that might potentially be effective in inhibiting toxin binding to receptors.

contd. In order to gain information on the location of the BTX-binding site on the primary sequence of the α -subunit of the AChR, ⁽¹²⁵⁾BTX binding to a 32 amino acid peptide (32-mer) comprising residues 173-204 of the Torpedo α -subunit was investigated. Toxin binds to the peptide with the same affinity as binding to the isolated α -subunit. Binding of toxin to the 32-mer could be competed with cholinergic antagonists. It is concluded that this region of the α -subunit is a major determinant of the toxin-binding site on the receptor. Investigation of toxin-binding to shorter peptides comprising portions of the 32-mer should further localize the site. Substitution of glutamine for the negatively-charged glutamate-180, which possibly represents the anionic site to which the quaternary ammonium group of acetylcholine and the guanidinium group of arginine-37 of the neurotoxins bind, in a calf 32-mer did not significantly affect toxin binding. Thus, either another negatively-charged residue is important in binding or, in view of the multisite interaction of BTX and AChR, alteration of a single residue has little effect on binding. Binding of ⁽¹²⁵⁾BTX was considerably less to corresponding calf and human 32-mers than to the Torpedo 32-mer. This raises the possibility that the neurotoxins do not have as great an effect on the human receptor and that other constituents of snake venoms contribute more to the toxic effects of the venoms in humans. Finally, antibodies could be raised against the Torpedo 32-mer. These antibodies cross reacted with membrane-bound and purified AChR, demonstrating the accessibility of the site on the intact receptor.

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SUMMARY

This research was designed to (1) develop binding-inhibition assays that could be used to screen compounds that might be effective in inhibiting neurotoxin binding to the acetylcholine receptor (AChR), (2) carry out studies to identify the anionic site on primary sequence of the α -subunit to which neurotoxins bind, and (3) determine if antibodies to synthetic receptor peptides might be effective in inhibiting neurotoxin binding to the receptor.

A solid phase radioassay was developed to measure the binding of neurotoxins to receptors using binding of ^{125}I -labeled α -bungarotoxin (BTX) to purified Torpedo AChR and membrane-bound AChR as a model. The solid phase assay is rapid, convenient, and utilizes small quantities of reagents. Association and dissociation rate constants and dissociation constants for BTX binding to the AChR determined from kinetic and equilibrium binding data ($K_D \rightarrow 10^{-11}$) were comparable to the constants observed with in vitro assays (filter disk and centrifugation assays) and to those reported in the literature, thus validating the solid phase assay. Besides α -bungarotoxin, a long, postsynaptic neurotoxin, binding of cobratoxin from Naja naja atra, a short, postsynaptic neurotoxin, and apamin, a centrally acting neurotoxin, could be demonstrated using the solid phase assay. The binding of apamin to brain synaptosomes indicates the assay is sensitive enough to be utilized with preparations containing a low density of receptors. The assay was also employed as a binding-inhibition assay by incubating constant amounts of toxin with increasing concentrations of cholinergic ligands (agonists and antagonists). This assay should be useful in screening compounds that might potentially be effective in inhibiting toxin binding to receptors.

In order to gain information on the location of the BTX-binding site on the primary sequence of the α -subunit of the AChR, ^{125}I -BTX binding to a 32 amino acid peptide (32-mer) comprising residues 173-204 of the Torpedo α -subunit was investigated. Toxin binds to the peptide with the same affinity as binding to the isolated α -subunit. Binding of toxin to the 32-mer could be competed with cholinergic antagonists. It is concluded that this region of the α -subunit is a major determinant of the toxin-binding site on the receptor. Investigation of toxin-binding to shorter peptides comprising portions of the 32-mer should further localize the site. Substitution of glutamine for the negatively-charged glutamate-180, which possibly represents the anionic site to which the quaternary ammonium group of acetylcholine and the guanidinium group of arginine-37 of the neurotoxins bind, in a calf 32-mer did not significantly affect toxin binding. Thus, either another negatively-charged residue is important in binding or, in view of the multisite interaction of BTX and AChR, alteration of a single residue has little effect on binding. Binding of ^{125}I -BTX was considerably less to corresponding calf and human 32-mers than to the Torpedo 32-mer. This raises the possibility that the neurotoxins do not have as great an effect on the human receptor and that other constituents of snake venoms contribute more to the toxic effects of the venoms in humans. Finally, antibodies could be raised against the Torpedo 32-mer. These antibodies cross reacted with membrane-bound and purified AChR, demonstrating the accessibility of the site on the intact receptor.



Yale University
Inter-Office Correspondence

Date:

To:

From:

Foreword

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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PROBLEM UNDER STUDY

The following three problems were studied during the term of this contract:

a. Assay Development. The suitability of solid phase radioassays to measure the binding of neurotoxins to membrane preparations or purified acetylcholine receptor was investigated. The toxins studied were α -bungarotoxin, cobratoxin, and apamin. Experiments were conducted to determine whether the solid phase assay could be used as a binding-inhibition assay to screen compounds that might inhibit binding of toxins.

b. Structure/Function Studies. Studies were performed in an effort to identify the site on the primary sequence of the α -subunit of the acetylcholine receptor to which curare-mimetic neurotoxins bind. A synthetic peptide of a portion of the α -subunit was synthesized and tested for ability to bind α -bungarotoxin. The specificity of binding was analyzed by competition experiments using cholinergic agonists and antagonists.

c. Therapy. Antibodies were raised against the α -subunit peptide because these might be effective in inhibiting neurotoxin binding to the receptor. Polyclonal antibodies were raised in rabbits and monoclonal antibody production undertaken using mice.

BACKGROUND AND REVIEW OF LITERATURE

The nicotinic acetylcholine receptor (AChR) functions to transduce a chemical signal (acetylcholine) into an electrical event by opening a channel through which sodium ions pass. The AChR has been well characterized, both physiologically and biochemically (1-3). The AChR purified from the electroplaque of the electric organ Torpedo californica consists of four subunits present in the molar stoichiometry of $\alpha_2\beta\gamma\delta$ (3-6). The Torpedo AChR has close structural and functional similarities with AChR from other sources including mammals (7). The pentameric complex of subunits contains the agonist binding site responsible for receptor activation and desensitization as well as the ion channel for cation flux through the membrane. Although the AChR is the best characterized neurotransmitter molecule, many questions concerning its function remain unanswered. These include the nature of the interaction of the transmitter with the receptor and how this leads to opening of the ion channel. Recent information on the primary amino acid sequences of the polypeptides comprising the receptor (8-11) provides a basis for analyzing and understanding the receptor functions at a molecular level.

The study of the AChR has been aided by the use of snake venom curare-mimetic neurotoxins. Neurotoxins from snakes belonging to the families Elapidae (cobras, kraits, mambas, and others) and Hydrophidae (sea snakes) are polypeptides of 60-74 residues which bind with high affinity to the nicotinic AChR (12,13), and as with curare, competitively block the depolarizing action of acetylcholine (14). The binding of toxins is competitive with the binding of the affinity alkylating agents 4-(N-maleimido)- α -benzyltrimethylammonium (MBTA) and bromoacetylcholine (1). α -Bungarotoxin (BTX), a long neurotoxin, binds with high affinity to the Torpedo AChR (12-15). The most widely used procedure for measuring toxin-binding to the AChR is the DEAE-cellulose disk assay (filter disk assay) of Schmidt and Raftery (16). BTX binds with lowered affinity to isolated α -subunit but not to β , γ , and δ subunits (17-19). The

competition between cholinergic agonists and neurotoxins suggests that the toxin-binding region of the α -subunit includes the ACh-binding site.

The snake venom neurotoxins are flat, hand-shaped molecules with three polypeptide loops projecting from a central core of four disulfide bridges near one end (20-28). All of the side chain residues that have been identified as being important functionally are located on the concave surface of the molecule which comes into contact with the AChR (28). The residues which interact with the acetylcholine-binding site on the AChR lie at the end of neurotoxin loop 2, the so-called toxic loop, protruding from the molecule. The cationic guanidinium group of the invariant arginine-37 of the neurotoxin is considered to be the counterpart of the quaternary ammonium group of acetylcholine (20-23). Most likely, an anionic site on the AChR lying beneath arginine-37 of the toxins forms part of the acetylcholine-binding site (28). Other toxin residues participate in binding to the AChR (28,29) and such multipoint interactions may be responsible for the high affinity of toxin binding (30).

Several models of the secondary structure of the α -subunit of the AChR have been proposed (11,28,31-35). These differ largely in the number of membrane-spanning α -helices. The acetylcholine binding site lies on the ectodomain of the α -subunit. The binding site is located near the disulfide group which is reduced and alkylated by the affinity ligands. Based on the size of these ligands, the negative subsite binding the quaternary ammonium group is about 10Å from the sulfhydryl group formed by reduction of the disulfide (1). The α -subunit has four cysteine residues which can form two disulfides. Initially, a disulfide occurring between cysteine residues at positions 128 and 142 was postulated as the most likely region of the acetylcholine-binding site (36). An alternative region is near cysteine residues at positions 192 and 193. These cysteines are present in the α -subunit but not the other subunits. Karlin and co-workers have shown that cysteine-192, and possibly in addition cysteine-193, were specifically labeled by the affinity alkylating agent MBTA (37). In addition, evidence has been presented that a disulfide bond exists between cysteine 192 and 193 (38). Thus, it seems likely that at least a portion of the acetylcholine binding site lies near cysteine 192 and 193. It is possible that other regions of the α -subunit brought into proximity to this region contribute to the binding site. Recently, a synthetic peptide comprising residues 173-204 of the α -subunit has been shown to bind BTX with the same affinity as the isolated α -subunit (39). This finding suggests a major determinant of the BTX-binding site in the α -subunit resides between residues 173 and 204. Furthermore, a shorter peptide, residues 185-196, has also been shown to bind BTX, although with lower affinity (40).

RATIONALE USED IN CURRENT STUDY

a. Assay Development. As noted above, BTX has been extremely useful in investigating the AChR because of its specificity and high affinity. A filter disk assay (16) has been widely employed to measure BTX binding to the AChR. However, in preliminary experiments, we have found that toxin binding can be measured using solid phase radioassays in which purified receptor is adsorbed to the wells of polystyrene microtiter plates and the binding of ^{125}I -labeled BTX measured. The solid phase radioassay is considerably easier to perform than the filter disk assay. In addition, it lends itself to performance of binding inhibition assays in which a potential inhibitor is added along with

¹²⁵I-BTX and effect on binding measured. Therefore, it was planned to develop a model protocol for solid phase radioassays and binding-inhibition assays using ¹²⁵I-BTX and receptor-rich membranes and purified AChR from Torpedo electric organ. In order to validate the solid phase assay, the kinetics and binding constants determined with this assay were compared with those obtained with in vitro assays (the filter disk assay and centrifugation assay). In addition, experiments were performed to determine whether the solid phase assays can be applied to other neurotoxins. In this case, the solid phase assays could have wide applicability in screening compounds that would potentially inhibit toxin binding. Different classes of toxins were selected for investigation. Erabutoxin is a short, postsynaptic neurotoxin compared to BTX, which is a long, postsynaptic neurotoxin. β -Bungarotoxin is also a component of snake venoms, but acts presynaptically to inhibit transmitter release. Cardiotoxin is another component of snake venoms, but interacts with the membranes of other cells such as muscle to produce persistent depolarization. Apamin is a neurotoxic octadecapeptide from bee venom which, unlike other peptide toxins, crosses the blood-brain barrier to exert effects on the central nervous system.

b. Structure/Function Studies. These studies were designed to identify the BTX and hence acetylcholine binding site on the AChR. Because the toxin is competitive with acetylcholine binding, the toxin-binding site must be located at or very near the acetylcholine-binding site, and therefore identification of the toxin-binding site should also localize the acetylcholine binding site. Because the primary amino acid sequence of the α -subunit is known, it is possible to synthesize peptides comprising portions of the α -subunit and to test these for ability to bind BTX. Evidence exists that studies of such ligand-peptide interactions are feasible. A 33 residue synthetic peptide containing loop 2 of Naja naja philippinensis toxin has been shown to bind to the AChR and to compete with BTX for binding (41). In addition, BTX has been shown to bind to proteolytic fragments of the α -subunit as small as 7 kD in molecular mass (42). Finally, 32-residue (39) and 12-residue (40) peptides of the α -subunit have been shown to bind BTX. Thus, neither the entire toxin or entire α -subunit is necessary for binding. The fact that synthetic peptides bind BTX does not provide conclusive evidence that the acetylcholine binding site is also located on the peptide. It is possible that the peptides interact with some portion of the toxin molecule other than that binding to the acetylcholine binding site. To determine whether the acetylcholine binding site resides on the peptide, it is necessary to determine whether the binding of BTX is competed with cholinergic antagonists or agonists. Therefore, in these studies, the 32-residue peptide (residues 173-204) was synthesized and the ability of cholinergic antagonists to inhibit BTX binding tested. The 32-mer contains several negatively-charged residues that represent candidates for the anionic subsite. To further localize the binding site, shorter peptides comprising portions of the 32-mer were synthesized with the goal of testing them for ability to bind toxin.

c. Therapy. If the toxin binding site of the receptor can be identified, a basis is provided for developing therapies to inhibit binding of the toxin to the receptor and thus toxicity. Highly specific homogeneous toxin-like probes directed at many parts of the AChR molecule can be obtained from cloned hybridoma cell lines producing monoclonal antibodies (43). These have had many useful applications in the study of the AChR and the auto-immune response to the receptor in myasthenia gravis (44). It can be expected that antibodies to the toxin-binding peptide and the synthetic receptor peptide itself should compete or block binding of toxin to the receptor. Thus, anti-

bodies were raised against the 32-residue peptide in rabbits. The production of monoclonal antibodies in mice was undertaken. These antibodies can then be tested for ability to block toxin binding to AChR using the solid phase radioassay and for ability to prevent toxicity in vivo.

EXPERIMENTAL METHODS

General Procedures

Torpedo electric organ membranes. Frozen electric organ tissue (50-100g) from Torpedo californica (Pacific Biomarine, Venice, CA) was minced and homogenized in 50-100-ml of homogenization buffer (0.4 M Tris HCl, pH 7.4, 10 mM Na₂ EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% NaN₃), for 2-3 min with a Virtis homogenizer. After centrifugation in a Sorvall RC-5 centrifuge (10 min at 4,000 xg), the supernatant was centrifuged at 100,000 xg for 45 min in a Beckman L2-65 ultracentrifuge with a 50.2 Ti rotor. The membrane pellet was resuspended and recentrifuged at 100,000 xg. The final membrane pellet was resuspended in homogenization buffer to give a protein concentration of ~ 10 mg/ml. This fraction was stored at -70°C until use.

Torpedo AChR. The AChR was affinity-purified on a cobratoxin-Sepharose column essentially as described by Froehner and Rafto (45). Protease inhibitors (0.1 mM PMSF and 10 units Trasylol/ml) and divalent cation chelators (1 mM EDTA and 1 mM EGTA) were present throughout the extraction. Torpedo electric organ membranes were solubilized in Triton X-100 and passed over a cobra-toxin-Sepharose column. The AChR was eluted from the column with 1 M carbamylcholine (carbachol) in 50 mM Tris-HCl, pH 7.4, 0.1% deoxycholate. The purified AChR was dialyzed against the same buffer containing the protease inhibitor. The receptor preparations routinely bound ~ 7.5 nmol of ¹²⁵I-labeled α -BTX per mg of protein. Upon sodium dodecyl sulfate (SDS), polyacrylamide gel electrophoresis (PAGE), the four receptor subunits were revealed, demonstrating the purity of these preparations.

Synaptosome preparation. Five to 10 brains from adult male Sprague-Dawley rats were homogenized in 0.32 M sucrose in 5 mM Tris-HCl, pH 7.4, protease inhibitors, 10% w/v, with a glass homogenizer. The homogenate was centrifuged at 1000 xg and the pellet resuspended and washed twice. The supernatants were combined and centrifuged at 23,000 xg for 20 min in a L2-65 ultracentrifuge with a Ti 45 rotor. The pellet was resuspended in a small volume of 0.32 M sucrose in 5 mM Tris-HCl, pH 7.4, layered on a discontinuous sucrose gradient (0.8, 1.0, 1.2 M) and centrifuged at 33,000 xg for 60 min in a SW41 rotor. The material at the 1.0 M and 1.2 M interface was removed with a J syringe, added to PBS (1:1), and centrifuged at 100,000 g in a 50.2 Ti rotor for 30 min. The pellets were resuspended in PBS, recentrifuged, resuspended, aliquoted, and protein concentration determined by Lowry.

Iodination of BTX and other proteins. BTX was iodinated using the chloramine T method (46) essentially as described by Wang and Schmidt (47). The iodinated mixture was chromatographed on a Sephadex G-10 column to remove free iodide. The iodinated toxin was then fractionated on a CM-Sephadex column to separate di- and monoiodinated BTX. The latter was used in these studies. Other toxins and the 32-mer were iodinated in the same manner.

Toxins. Toxins were obtained from the following sources:

α -Bungarotoxin: Miami Serpentarium
 α -Cobratoxin (*Naja naja siamensis*): Miami Serpentarium
Cobratoxin (*Naja naja atra*): Miami Serpentarium
Erabutoxin: Sigma Chemical Co.
 β -Bungarotoxin: Miami Serpentarium
Cardiotoxin: Sigma Chemical Co.
Apamin: Sigma Chemical Co.

All other reagents were analytical grade from conventional commercial sources.

Assay Development

Solid phase radioassay. A protocol was developed for measuring the binding of iodinated toxins to purified receptor or membranes adsorbed to the wells of polystyrene microtiter plates. A large number of preliminary experiments were performed to determine the optimum conditions and amounts of material to be used. The protocol developed to measure binding of BTX to Torpedo membranes and purified AChR is described below. This assay was also utilized to measure binding of erabutoxin and cobratoxin to the AChR, β -bungarotoxin to synaptosomes, and apamin to synaptosomes. To characterize binding of toxins, experiments were performed to measure toxin binding as a function of receptor concentration, the time course of association and dissociation, and saturability of binding. Competition experiments were performed by incubating a constant amount of labeled toxin with increasing amounts of competitor and measuring bound radioactivity. The effects of unlabeled BTX, α -cobratoxin, d-tubocurarine, nicotine, carbachol, choline, and NaCl on ^{125}I -BTX binding were tested. The conditions of each individual experiment are described in the figure legends in the Results section.

SOLID PHASE RADIOASSAY

α -Bungarotoxin to Acetylcholine Receptor

1. Make up receptor or membranes at desired concentration (1-10 $\mu\text{g/ml}$ for receptor, 10-25 $\mu\text{g/ml}$ for membranes) in coating buffer (0.05 M carbonate/bicarbonate buffer, pH 9.6 containing 0.02% NaN_3).
2. Add 100 μl (0.1-2.5 μg) of receptor or membrane solution to wells of 96-well polystyrene microtiter plates (Nunc, USA/Scientific Plastics). Use 3-4 replicates for each condition. Add coating buffer to other wells for background measurements. Experimental and control wells are treated identically in subsequent steps.
3. Incubate overnight at 4°C in humidified chamber (plastic box with wet paper towel) to adsorb receptor or membranes to plastic. Alternatively, plates containing membranes can be centrifuged at $\sim 1,000 \times g$ in plate carriers for 30 min.
4. Wash 3 times with PBS (10 mM phosphate buffer, 0.15 M NaCl, pH 7.4). Wells can be aspirated with a Pasteur pipette and a vacuum source. PBS is dispensed with a 12-channel Titertek dispenser.

5. Quench wells by adding 300 μ l of 5% BSA (Sigma, Fraction V) in PBS.
6. Wash wells 3 times with PBS.
7. Add 100 μ l 125 I-labeled α -bungarotoxin (*BTX) to wells. The buffer, amount of toxin, length of incubation, etc., depend on the specific activity of the *BTX and nature of the experiment. Routine conditions are 1-5 nM *BTX (25,000-100,000 cpm/well) in 10 mM phosphate buffer, pH 7.
8. For competition experiments, add 50 μ l of competitor in increasing concentrations to wells. Immediately add 50 μ l of *BTX at constant concentration (1-5 nM). (Note that final concentration of each will be half the starting concentration.) Incubate \sim 10 min (remain in linear phase of association of *BTX).
9. Wash 5 times with PBS.
10. To remove and measure bound radioactivity, remove buffer and add 200 μ l 5% SDS in 0.2 N NaOH. Let stand 2-5 min. Remove solution from each well with a Pipetteman and place in a gamma vial or tube. Repeat twice. Count in gamma counter. Subtract cpm in control wells from cpm in experimental wells.

Note: This protocol can be used for many ligand-receptor interactions. Ligands include labeled monoclonal antibodies, IgG, protein A, Con A, hormones, toxins, peptides, viruses, etc. If the receptor is present in low concentration, use 24-well culture plates (Costar), the wells of which have a greater surface area. Two-step procedures employing a primary antibody and a labeled second antibody or protein A can be used. It is important to determine the optimum conditions for each protocol as they will differ to some degree. This is achieved by performing test experiments in which the concentration of reagents used and time of incubation for each step (receptor or membrane, quench, and ligand) is varied independently. If backgrounds are high, increase the quench concentration (to 10% BSA), number of washes, or include detergent in the washes (0.02 to 0.1% Triton X-100 or Tween 20). For viruses, especially, inclusion of Triton in the final wash step reduces the background greatly. For ligands not easily solubilized (e.g., viruses), place the plate containing SDS-NaOH on a hot plate or in an oven (\sim 45°C).

The amount of receptor that actually bound to the plastic after adsorption was determined. To wells, 0.4 μ g (400 fmol) of AChR was added. After washing and quenching for 50 min with 5% BSA, wells were incubated with an excess of 125 I-BTX (3.6×10^{-8} M, 331 cpm/fmol). Background binding of 125 I-BTX to quenched wells lacking AChR was measured and subtracted from experimental values. Under equilibrium conditions, 24,105 cpm or 73 fmol of 125 I-BTX were bound. Because there are 2 α -subunits per receptor, the toxin binding sites represent 36.5 fmol AChR, or 9% of the total AChR added to the wells.

To validate the results obtained with the solid phase assay, BTX binding to the AChR was also measured using *in vitro* assays in which 125 I-BTX is incubated with receptor or membranes in solution. Thus, the toxin-receptor complex is separated from unbound toxin by immobilization on a filter in the filter disk assay or centrifugation in the centrifugation assay. The proto-

cols for these assays are shown below. Experiments comparable to those performed with the solid phase assay to determine the kinetics of binding were carried out and the results compared. Dissociation constants were calculated as described by Bylund (48).

DEAE - CELLULOSE FILTER DISK ASSAY

The amount of toxin and receptor, length of incubation, amount of buffer etc. depend on the specific activity of the hot toxin, the number of receptor sites, the nature of binding, and the type of experiment performed. Routine conditions are nanomolar toxin concentrations. Filters must be tested with hot toxin alone to determine the nonspecific background. This depends on the overall charge of the toxin.

1. Make up AChR in 50 mM PB (phosphate buffer) + 0.1% Triton X-100 + 0.005% NaN_3 , pH 6.5-7.5. Concentration of receptor usually should be $3-6 \times 10^{-3}$ $\mu\text{g}/\mu\text{l}$.
2. Make up toxin in 50 mM PB + 0.1% Triton X-100 + 0.005% NaN_3 + 0.2% BSA, pH 7.0.
3. Add toxin and receptor to 2 glass tubes. In 1 tube place 500-1000 fold excess of cold toxin. Put the corresponding volume of buffer in the other tube. Both tubes are treated identically in subsequent steps. Vortex each tube.
4. Incubate for desired time.
5. Spot 50 μl on a filter (Whatman DE-81, 25 mm diameter). Do not allow to dry.
6. Wash filters in washing buffer (PBS + 0.1% Triton) for 10 min with gentle shaking. Do not use a stir bar or over-agitate. Filters will disintegrate. Repeat twice more.
7. Count filters in a gamma counter.

CENTRIFUGATION ASSAY

1. Make up membranes at desired concentration in 10 mM PB + 0.005% NaN_3 , pH 7.4.
2. Make up ^{125}I -labeled toxin. The buffer, amount of toxin, length of incubation, etc., depend on the specific activity of the hot toxin and the nature of the experiment. Routine conditions are 1-5 nM labeled toxin (10,000-150,000 cpm/tube) in 10 mM PB + 0.2% BSA + 0.005% NaN_3 , pH 7.0 (BSA necessary in buffer).
3. Quench microcentrifuge tubes (polypropylene, USA/Scientific Plastics) by adding 500 μl of 5% BSA in PB. Spin 10 min in microfuge ($\approx 13,000 \times g$), aspirate supernatant.

4. Add 500 μ l (0.0025 μ g-10 μ g) of membrane to each microcentrifuge tube. Use three replicates for each condition. To determine nonspecific binding of toxin to tube, run controls without membranes (some toxins "stick" to plastic more than others). Experimental and control tubes are treated identically in subsequent steps. Spin 10 min, aspirate supernatant.
5. Add 250-500 μ l labeled toxin to each tube (the minimum volume is 250 μ l). Vortex. Incubate until equilibrium is reached. For association experiments the reaction is stopped with the addition of excess cold toxin (200-1000 X excess). Spin 10 min, aspirate supernatant (for saturation experiments save 50 μ l of supernatant to determine the concentration of free toxin).
6. Wash with 1000 μ l PBS, vortex. Spin 10 min, aspirate. Repeat three times.
7. Remove sample with 500 μ l NaOH + SDS and count in a gamma counter.

Structure/Function Studies

Synthetic peptides. Peptides comprising portions of the α -subunit of the AChR were synthesized by the Protein Chemistry Facility of the Department of Molecular Biophysics and Biochemistry, Yale University. The sequences of the peptides synthesized are shown in Table 1. Toxin binding to peptides was tested according to the protocol listed below:

125 I-BTX Binding to 32-mer

1. Coating: 5 μ g 32-mer were added to the wells of microtiter plates in 100 μ l of coating buffer (0.05 M carbonate/bicarbonate buffer, pH 9.6, containing 0.02% NaN_3) and incubated overnight at room temperature.
2. Quench: Peptide is removed and wells quenched by adding 2% BSA in 10 mM phosphate buffer, pH 7.5, for 1 h at room temperature.
3. Incubation: Quench is removed and 1 nM 125 I-labeled BTX in 0.1% BSA, 10 mM phosphate buffer, pH 7.5, added to wells and incubated 2 h at room temperature.

Competition experiments are performed by adding increasing concentrations of competitor to wells followed by 1 nM 125 I-BTX.

4. Wash: Wells are washed 4 times with 200 μ l of 0.1% BSA in 10 mM phosphate buffer.
5. Solubilization: Radioactivity is removed by adding 100 μ l of 2.5% SDS in 0.25 N NaOH, swabbing well with cotton-tipped applicator, placing applicator in a vial and counting in a gamma counter.

TABLE 1

Sequence and Alignment of Synthetic Peptides of AChR α -Subunit

<u>Torpedo</u> 32-mer	173SGEWMKDYRGWKHWVYYTCCPDTPYLDITYH 204
Human 32-mer	SGEWWIKESRGWKHSVTYSCCPDTPYLDITYH
Calf 32 mer	SGEWWIKESRGWKHWVFYACCPSTPYLDITYH
Modified Calf 32-mer	SGEWWIKQSRGWKHWVFYACCPSTPYLDITYH
<u>Torpedo</u> 19-mer	SGEWMKDYRGWKHWVYYT
<u>Torpedo</u> 12-mer	CPDTPYLDITYH
<u>Torpedo</u> 11-mer	PDTPYLDITYH
<u>Torpedo</u> 14-mer	KDYRGWKHWVYYTC
<u>Torpedo</u> 12-mer	KHWVYYTCCPDT
<u>Torpedo</u> 11-mer	HWVYYTCCPDT

A second assay was devised to demonstrate binding of peptides to BTX. In this assay, BTX is bound to plastic and incubated with purified AChR or 32-mer iodinated by the chloramine T method. The ability of peptides (which should compete with receptor 32-mer for binding to adsorbed toxin) to inhibit ^{125}I -AChR binding or ^{125}I -32-mer is tested. This protocol is described below:

1. Coating: Wells are coated with 5 μg BTX in coating buffer (0.05 M carbonate/bicarbonate buffer, pH 9.6, 0.02% NaN_3) overnight at room temperature.
2. Quench: BTX is removed and wells quenched with 300 μl 2% BSA in distilled water for 1 h at room temperature.
3. Incubation: ^{125}I -AChR or ^{125}I -32-mer in 10 mM phosphate buffer, pH 7.0, 0.1% BSA, and 0.2% Triton X-100 with increasing amounts of unlabeled AChR or 32-mer are added to wells and incubated for 15 min at room temperature.
4. Wash: Wells are washed 4 times in 10 mM phosphate buffer, pH 7.0, 0.1% BSA, and 0.2% Triton X-100.
5. Solubilization: 100 μl of 2.5% SDS in 0.25N NaOH is added to wells, wells are swabbed with a cotton-tipped applicator, the applicator placed in a vial, and radioactivity measured in a gamma counter.

Therapy

Polyclonal antibodies. Antibodies were raised against the Torpedo 32-residue peptide. The procedure for antibody production is outlined below.

- 1st injection: Four pound New Zealand white rabbits were injected subcutaneously in the back with 50 nmols 32-mer in complete Freund's adjuvant.
- 1st boost: Two weeks after the first injection, 50 nmols 32-mer in complete Freund's adjuvant was injected, half subcutaneously on the back and half intramuscularly in a thigh muscle. Two weeks after injection rabbits were bled from an ear vein after anesthetizing the ear with Solarcaine.
- 2nd boost: Two weeks after the second injection, 10 nmols 32-mer in complete Freund's adjuvant was injected, half subcutaneously in the back and half intramuscularly in a thigh muscle. Two weeks after injection, rabbits were bled from an anesthetized ear vein.
- 3rd boost: Same as second.

Sera were screened for antibodies against the 32-mer and AChR (purified AChR and AChR rich Torpedo membranes). Antigen was adsorbed to wells of 96-well microtiter plates, washed and quenched with 5% BSA, incubated with sera, washed, incubated with ^{125}I -labeled Protein A. After washing, bound radioactivity was removed and counted in a gamma counter.

Monoclonal antibodies. Monoclonal antibodies were raised against the 32-residue peptide essentially as described by Tzartos and Lindstrom (43). C57 black mice were used as immunizing animals and as a source of feeder spleen cells. Mice were injected subcutaneously with 32-mer (50 nmol) emulsified in complete Freund's adjuvant. A booster injection was given subcutaneously one week later and intravenously after another week. Mice were sacrificed 4 days later. Prior to sacrifice, an orbital bleed was performed and the serum tested for reactivity against the 32-mer using enzyme-linked immunosorbent assay (ELISA). Spleen cells from positive animals were fused with the mouse non-secreting mouse myeloma line Ag 653 with the procedure of Kohler and Milstein (49). Spleen cells were mixed with myeloma cells (10^9) and 50% polyethylene glycol (PEG 1500) added. Cells were then diluted with culture medium and rediluted in hypoxanthine-aminopterin-thymidine (HAT) medium in Costar microtest wells. After 14 days, supernatants from wells with proliferating hybrids were assayed for the presence of anti-32-mer antibody. Cells from positive wells were cloned by serial dilutions in 0.25% agar (50).

RESULTS

Assay Development

Solid Phase Assay

α -Bungarotoxin. The binding of ^{125}I -labeled BTX to AChR-rich membranes from Torpedo electric organs and the affinity purified AChR was measured using a solid phase assay. The time course of association for ^{125}I -BTX binding to Torpedo membranes is shown in Fig. 1. Equilibrium is reached in about 3 hours. Toxin binding increases as a function of membrane adsorbed to wells of microtiter plates (Fig. 2). Saturation of BTX binding to membranes is shown in Fig. 3. Nonspecific binding represents binding in the presence of a 200-fold excess of cold toxin. This value was subtracted from the total cpm bound to obtain the specific binding. The data of the saturation experiment are expressed by Scatchard analysis in Fig. 4. The slope of this plot yields a K_D for toxin binding of $3.5 \times 10^{-10}\text{M}$.

Similar experiments were performed to characterize BTX binding to purified AChR using the solid phase assay. The time course of association of toxin binding to AChR is shown in Fig. 5. Equilibrium is reached at about the same time as BTX binding to Torpedo membranes. Toxin binding as a function of the amount of receptor placed in the wells is shown in Fig. 6. Binding of toxin can be detected after adding as little as 0.1 μg AChR to the wells.

Dissociation of the ^{125}I -BTX-receptor complex was measured in the presence of a large excess of cold toxin (Fig. 7). Association and dissociation rate constants were calculated from the data in Figs. 5 and 7, as described by Bylund (48). The association rate (K_{+1}) is $3.45 \times 10^6 \text{ min}^{-1}\text{M}^{-1}$ and the dissociation rate constant (K_{-1}) is $1.5 \times 10^{-4} \text{ min}^{-1}$. The K_D calculated from the rate constraints is $4.3 \times 10^{-11}\text{M}$. Saturation of ^{125}I -BTX binding to AChR is shown in Fig. 8. Scatchard analysis of the data is illustrated in Fig. 9. The K_D calculated from the equilibrium binding experiment is $4.1 \times 10^{-10}\text{M}$.

Cobratoxin. The solid phase assay was also employed to study the binding of β -bungarotoxin, erabutoxin, cobratoxin (Naja naja atra) (CTX), and apamin. Specific binding (binding competed by excess cold toxin) was not detected in

BTX-TORPEDO ASSOCIATION

Solid Phase Assay

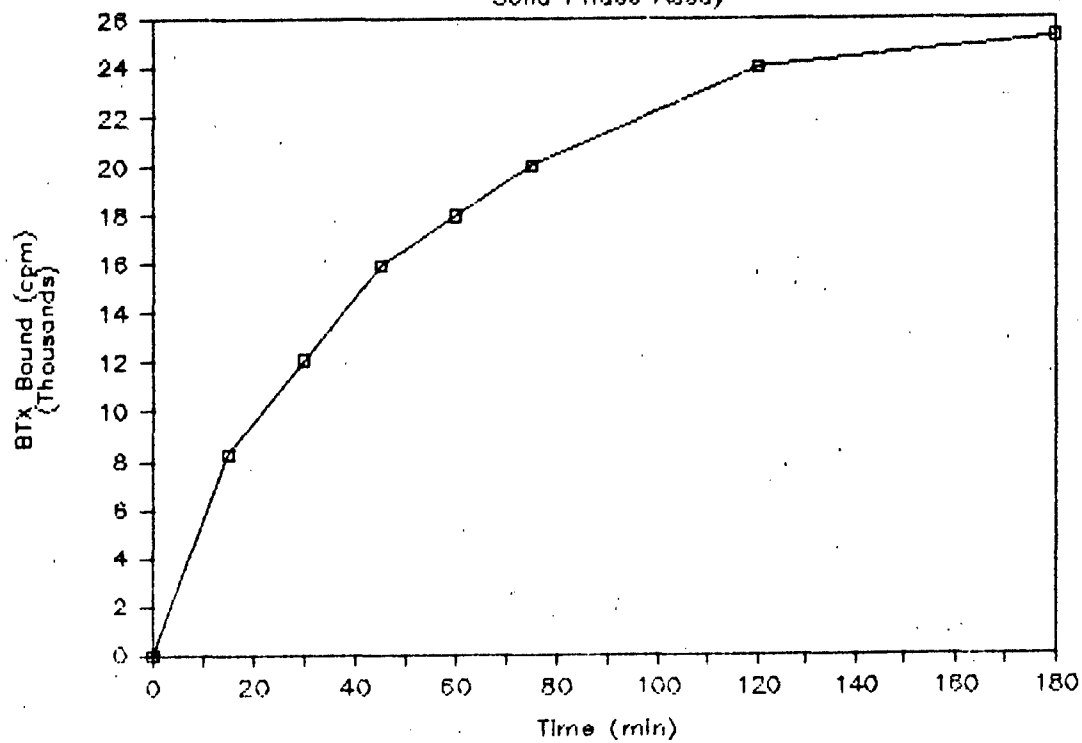


Fig. 1. Association of ¹²⁵I-BTX with AChR-rich membranes from Torpedo electric organ. Solid phase assay. Wells of microtiter plates were coated with 25 μ g membrane protein, quenched with 10% BSA, and incubated with 5.6×10^{-9} M ¹²⁵I-BTX for increasing periods of time. After washing, bound toxin was removed and counted in a gamma counter. Values are the mean of four replicates.

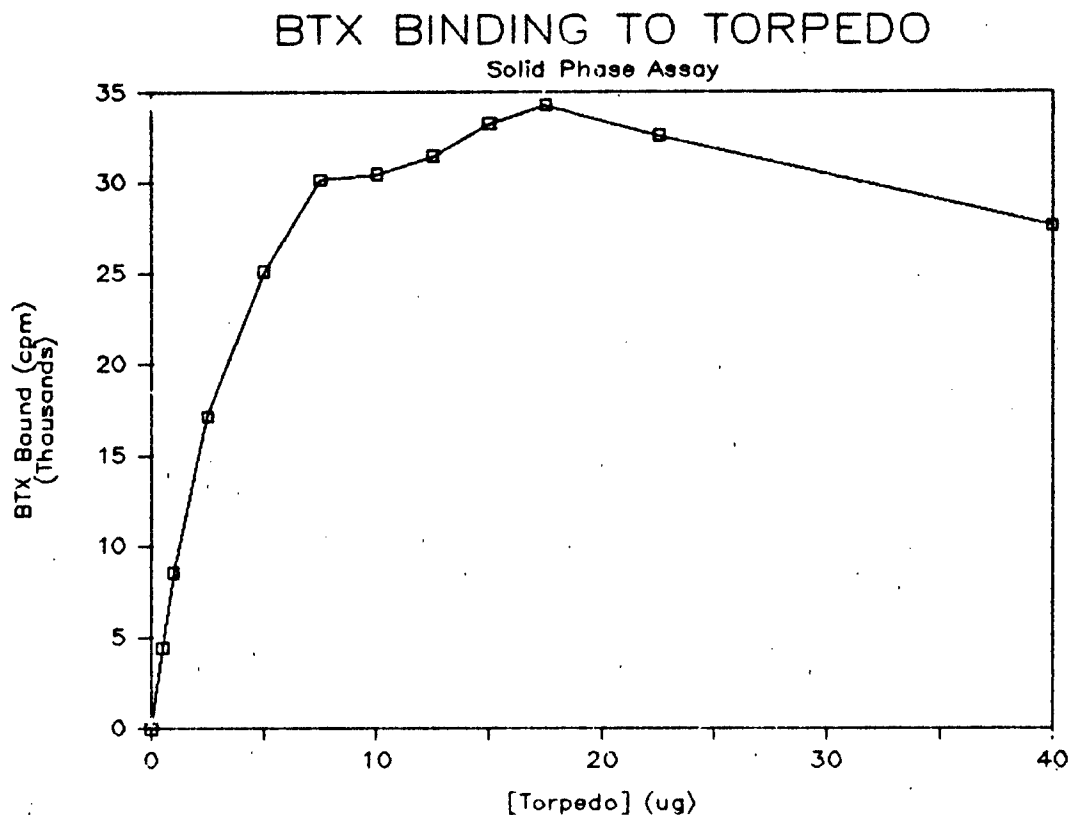


Fig. 2 ^{125}I -BTX binding to receptor rich membranes from Torpedo electric organ as a function of membrane concentration. Wells of microtiter plate were coated with increasing concentrations of Torpedo membrane protein. After quenching with 10% BSA, ^{125}I -BTX (5.6×10^{-9} M) was incubated for one hour, wells washed, and bound radioactivity removed and counted in a gamma counter. Values are the mean of four replicates.

SATURATION OF BTX BINDING TO TORPEDO

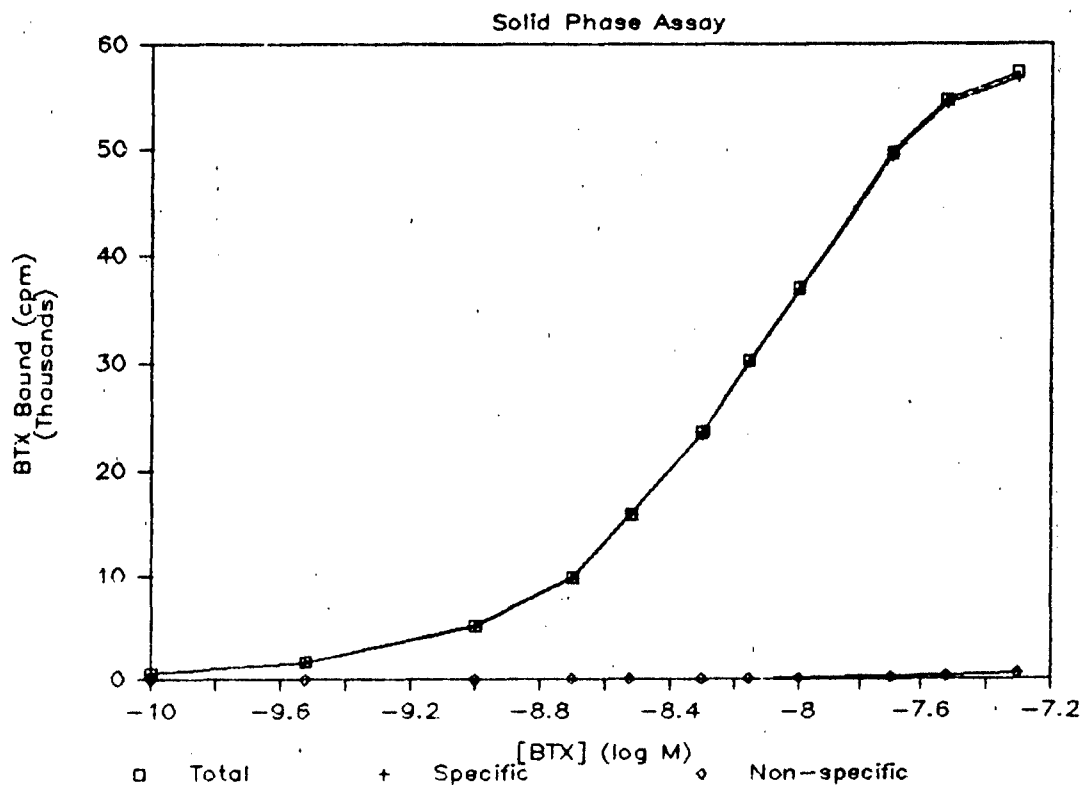


Fig. 3 Saturation of ^{125}I -BTX binding to receptor rich Torpedo membranes. Solid phase assay. Wells were coated with 10 μg membrane protein, quenched with 10% BSA, and incubated with increasing concentrations of ^{125}I -BTX ($1 \times 10^{-10} \text{ M}$ - $5 \times 10^{-8} \text{ M}$) for 2 hours. After washing, bound radioactivity was removed and counted in a gamma counter. Values are the mean of four replicates.

SCATCHARD PLOT FOR BTX-TORPEDO

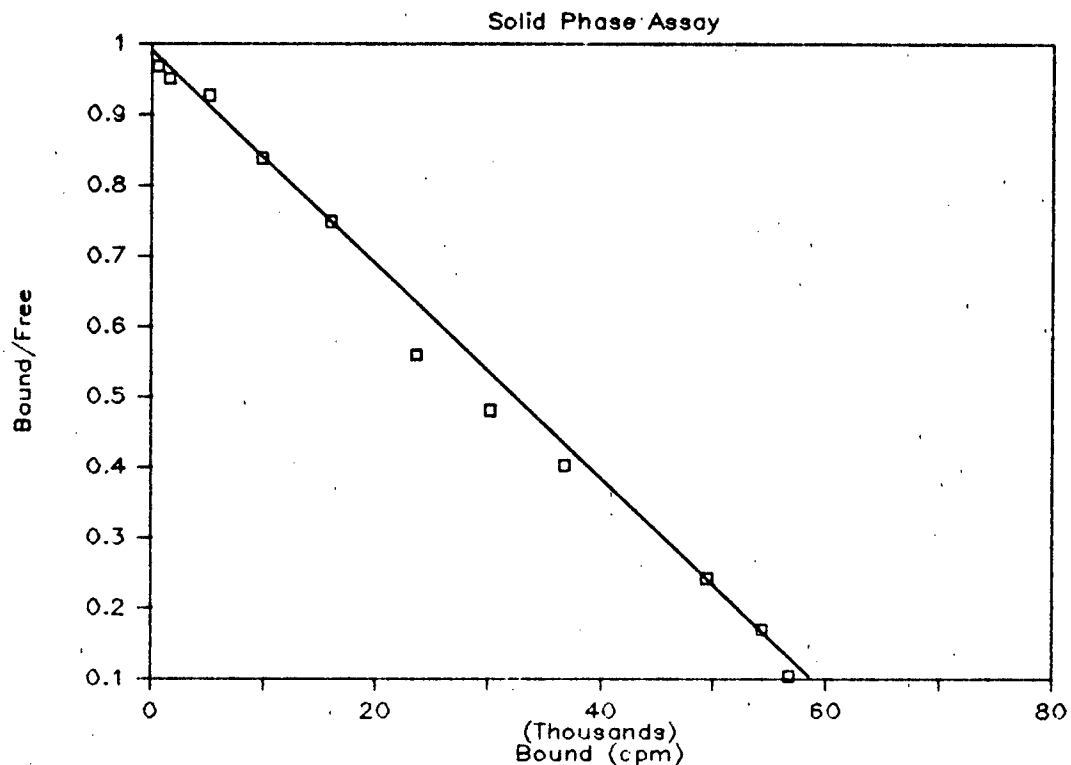


Fig. 4. Scatchard analysis of the data presented in Fig. 3. Bound toxin/free toxin was plotted versus bound toxin. The equilibrium K_D calculated from this data is 3.5×10^{-10} M.

BTX-AChR ASSOCIATION

Solid Phase Assay

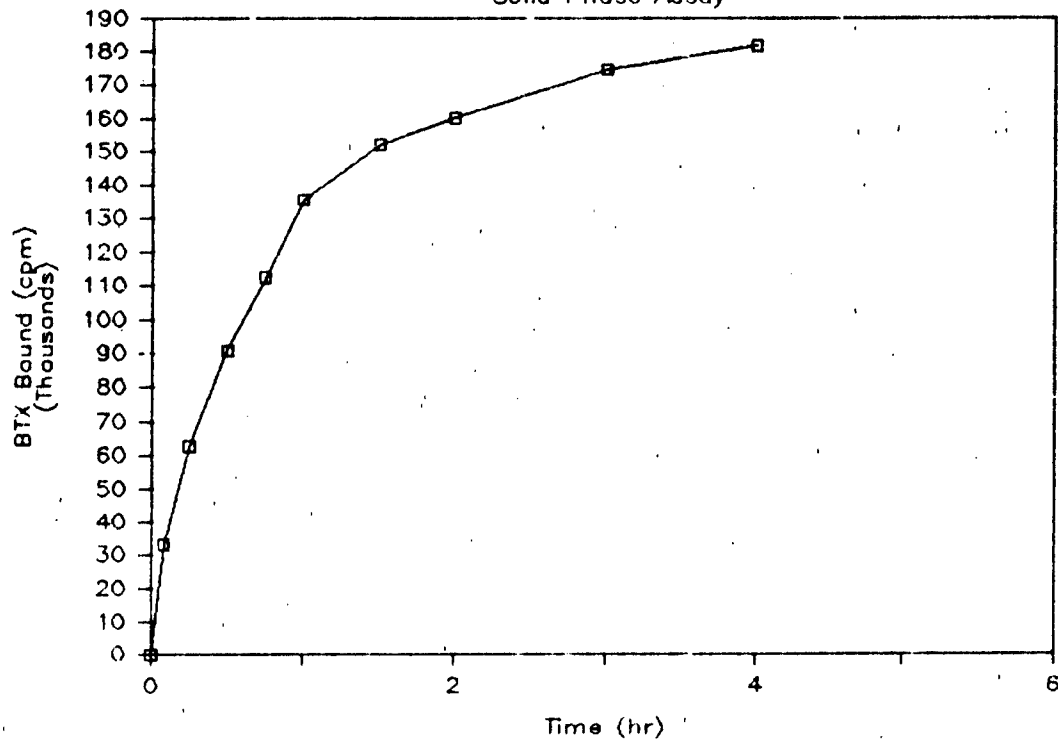


Fig. 5. Time course of association of ^{125}I -BTX with AChR. Solid phase assay. Wells of microtiter plates were coated with $0.2\text{ }\mu\text{g}$ AChR in coating buffer at 4°C overnight. Wells were washed, quenched with 5% BSA, washed and incubated with 5 nM ^{125}I -BTX in 50 mM phosphate buffer, $\text{pH } 6.5$, containing 0.2% BSA for increasing times. Bound radioactivity was removed with SDS/NaOH and counted in a gamma counter. Values are the mean of three replicates.

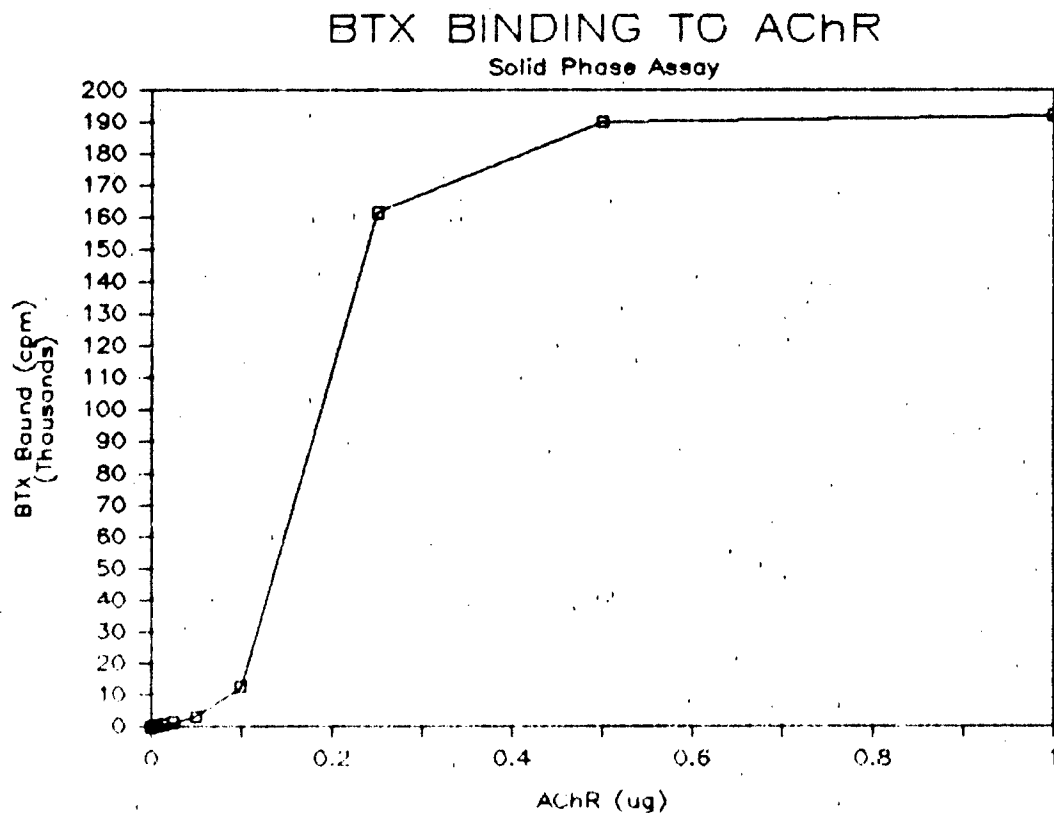


Fig. 6. ^{125}I -BTX binding to AChR as a function of receptor concentration. Solid phase assay. Wells of microtiter plates were coated overnight at 4°C with AChR in increasing concentrations in coating buffer. Wells were washed, quenched with 5% BSA, washed and incubated with 5nM ^{125}I -BTX in 50 mM phosphate buffer, pH 6.5, containing 0.2% BSA for 5 hours. After washing, bound radioactivity was removed and counted in a gamma counter.

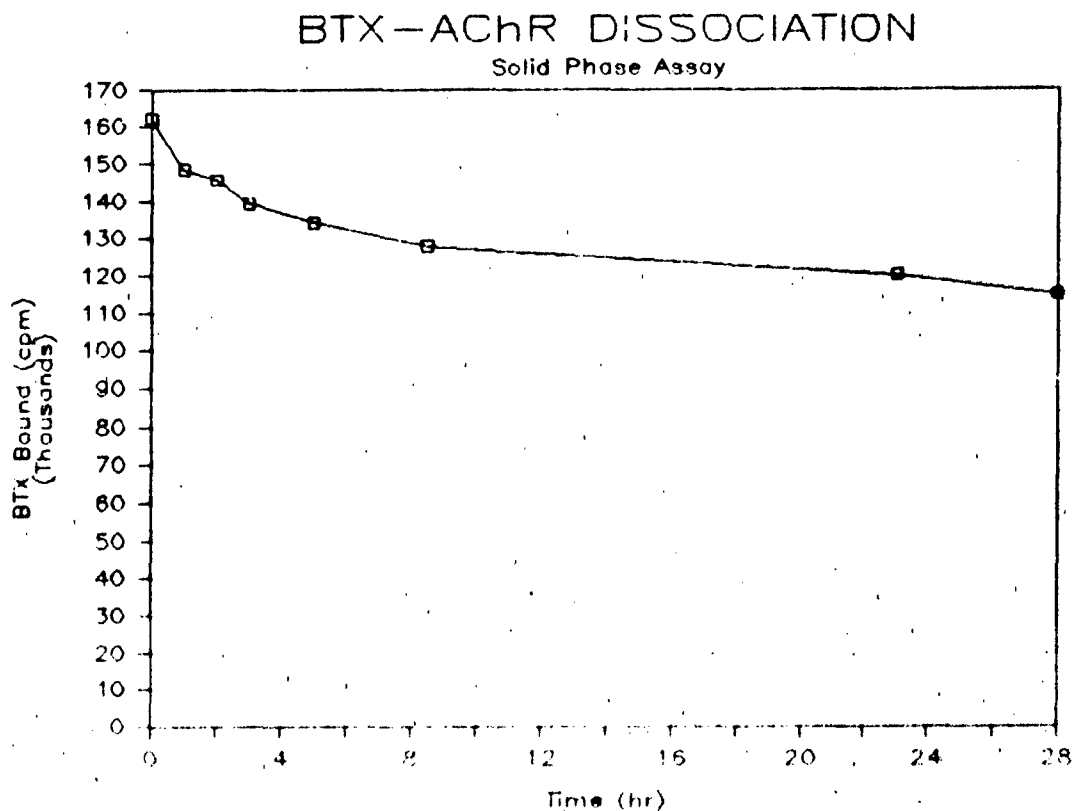


Fig. 7. Dissociation of ^{125}I -BTX from AChR. Solid phase assay. Wells of microtiter plates were coated with 0.2 μg AChR in coating buffer overnight at 4°C . Wells were washed, quenched with 5% BSA, washed and incubated with 5nM ^{125}I -BTX for four hours. After four hours, 1000-fold excess of cold BTX was added to the wells. At increasing intervals, wells were washed and bound radioactivity removed and counted in a gamma counter. Values are the mean of three replicates.

SATURATION OF BTX BINDING TO AChR

Solid Phase Assay

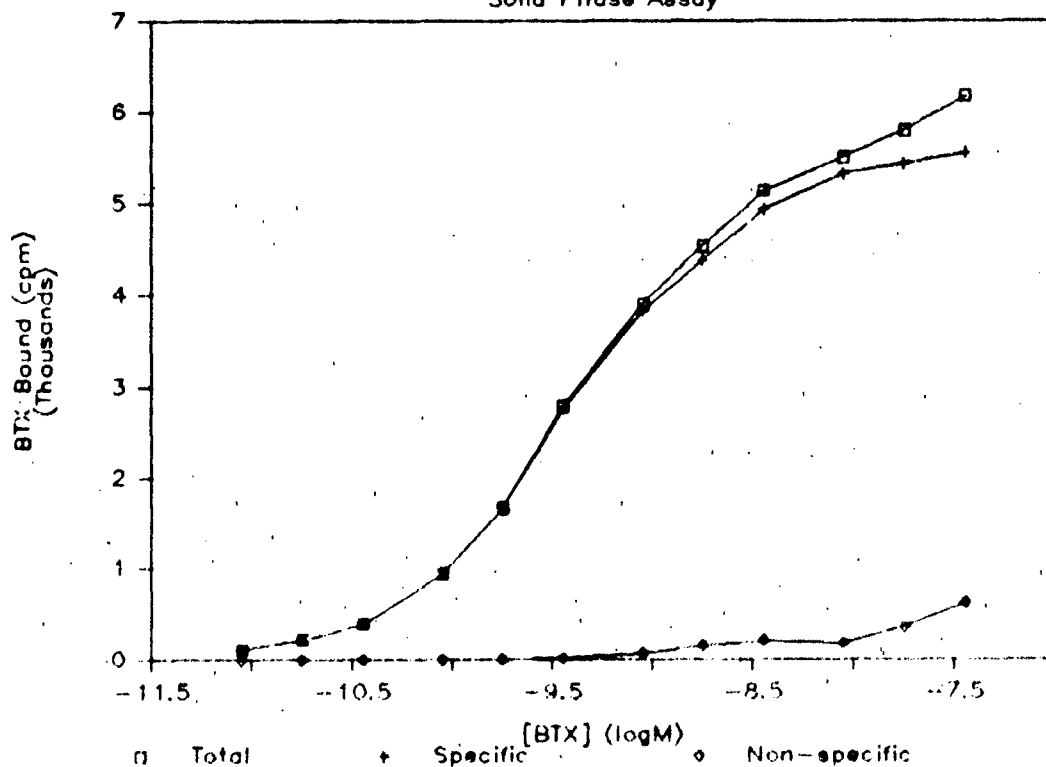


Fig. 8. Saturation of ^{125}I -BTX binding to AChR: Solid phase assay. Wells of microtiter plates were coated with 0.1 μg AChR in coating buffer overnight at 4°C . Wells were washed, quenched with 5% BSA for one hour, washed, and incubated with increasing concentrations ($2 \times 10^{-11}\text{M}$ - $5 \times 10^{-8}\text{M}$) of ^{125}I -BTX for five hours. Wells were washed and bound radioactivity removed and counted in a gamma counter. Non-specific binding is binding of ^{125}I -BTX in the presence of a 200-fold excess of cold BTX. This value was subtracted from the total radioactivity bound to determine the amount specifically bound. Values are the mean of three replicates.

SCATCHARD PLOT FOR BTX-AChR

Solid Phase Assay

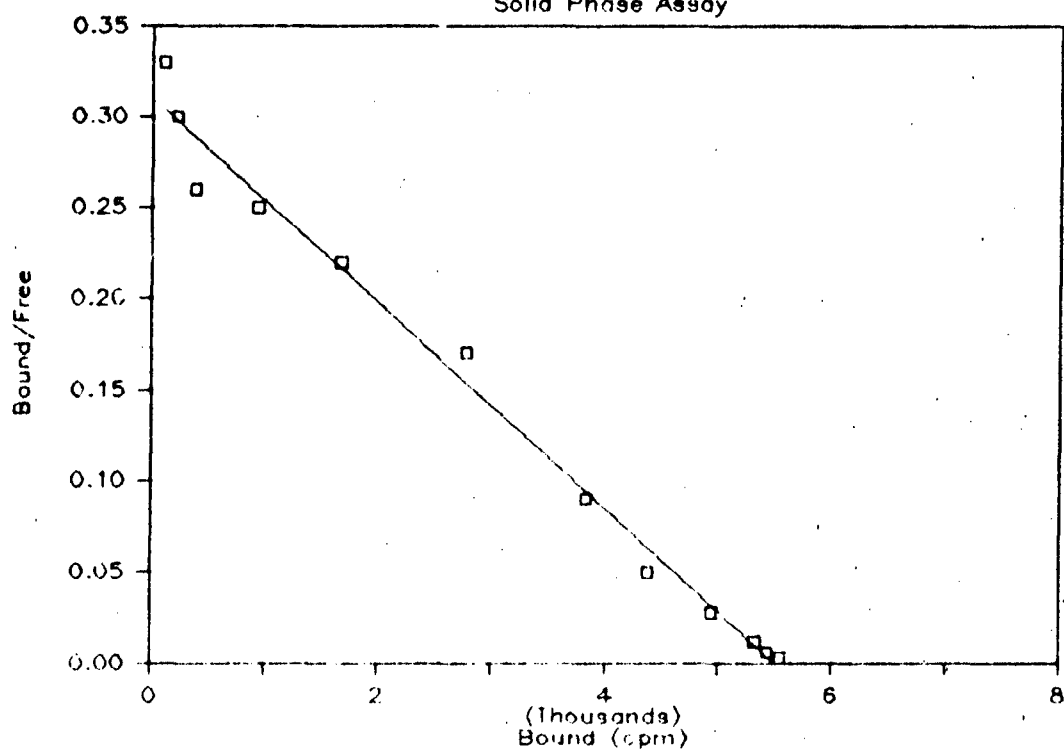


Fig. 9. Scatchard analysis of the equilibrium binding data in Fig. 8. Bound toxin/free toxin is plotted versus bound toxin. The equilibrium K_D calculated from this data is 4.1×10^{-10} M.

the case of β -bungarotoxin and erabutoxin. This was most likely due to inactivation of β -bungarotoxin by iodination and to inadequate iodination of erabutoxin. However, binding of cobratoxin, a short postsynaptic neurotoxin like erabutoxin, was observed.

Association (Fig. 10) and dissociation (Fig. 11) of CTX with AChR was measured. A biphasic curve was observed in the case of the dissociation experiment. Rate constants were calculated from the initial rates and are $K_{+1} = 1.2 \times 10^{-7} \text{ min}^{-1} \text{ M}^{-1}$ and $K_{-1} = 1.8 \times 10^{-3} \text{ min}^{-1}$. The K_D determined from these rate constants is $1.5 \times 10^{-10} \text{ M}$. Saturation of ^{125}I -CTX with the AChR was observed (Fig. 12). Scatchard analysis of this data is shown in Fig. 13. The K_D calculated from the equilibrium binding data is $2.7 \times 10^{-9} \text{ M}$.

Apamin. When ^{125}I -apamin binding to synaptosome membranes was tested using 96-well microtiter plates, only very low signals could be detected. However, adequate signals ($\sim 3,000 \text{ cpm}$) were obtained when synaptosomes were adsorbed to the wells of 24-well polystyrene plates (Costar) which have a considerably larger well area than the 96-well plates. Association of ^{125}I -apamin is shown in Fig. 14. It can be seen that equilibrium is reached in about 30 min. After that, there is a rapid decline in binding. This apparent loss of binding is most likely due to proteolytic digestion of either apamin or synaptosomes. Because of the loss of signal, it was not possible to perform a dissociation experiment. A saturation experiment in which synaptosomes were incubated with increasing amounts of labeled apamin was performed (Fig. 15). Saturation was not achieved because the labeled apamin was not present in high enough concentration. However, Scatchard analysis of the equilibrium binding data yielded a non-linear plot (Fig. 16). This is indicative of two binding sites of high and low affinity although it cannot be ruled out that the lower affinity binding represents non-specific binding.

Filter Disk Assay

α -Bungarotoxin. In the filter disk assay, ^{125}I -BTX is incubated with AChR in solution. An aliquot of the solution is then placed on filter paper and unbound ^{125}I -BTX, which does not adhere to the filter paper, is washed away and bound radioactivity measured. Using the filter disk assay, association (Fig. 17), dissociation (Fig. 18), and saturation experiments (Figs. 19, 20) were performed. One difference in comparison to the solid phase assay is that association is more rapid when measured with the filter disk assay, reaching equilibrium in 30 min. The K_{+1} is $9.3 \times 10^5 \text{ min}^{-1} \text{ M}^{-1}$ and the $K_{-1} = 4.1 \times 10^{-4} \text{ min}^{-1}$. The K_D determined from the rate constants is $4.4 \times 10^{-10} \text{ M}$. The K_D obtained from Scatchard analysis of the saturation data is $2.8 \times 10^{-10} \text{ M}$, comparable to the K_D obtained from equilibrium binding in the solid phase assay.

Centrifugation Assay

α -Bungarotoxin. Binding of ^{125}I -BTX to receptor-rich Torpedo membranes was studied using a centrifugation assay for comparison with results obtained using the solid phase assay. In the centrifugation assay, labeled toxin and membranes are incubated in solution, the membranes are pelleted by centrifugation to separate them from unbound labeled toxin, and bound radioactivity in the pellet measured. Association of toxin with membranes is illustrated in Fig. 21. The rate of association is more rapid than that observed with the solid phase assay (Fig. 1). Saturable binding of toxin to membranes was observed (Fig. 22).

CTX-AChR ASSOCIATION

Solid Phase Assay

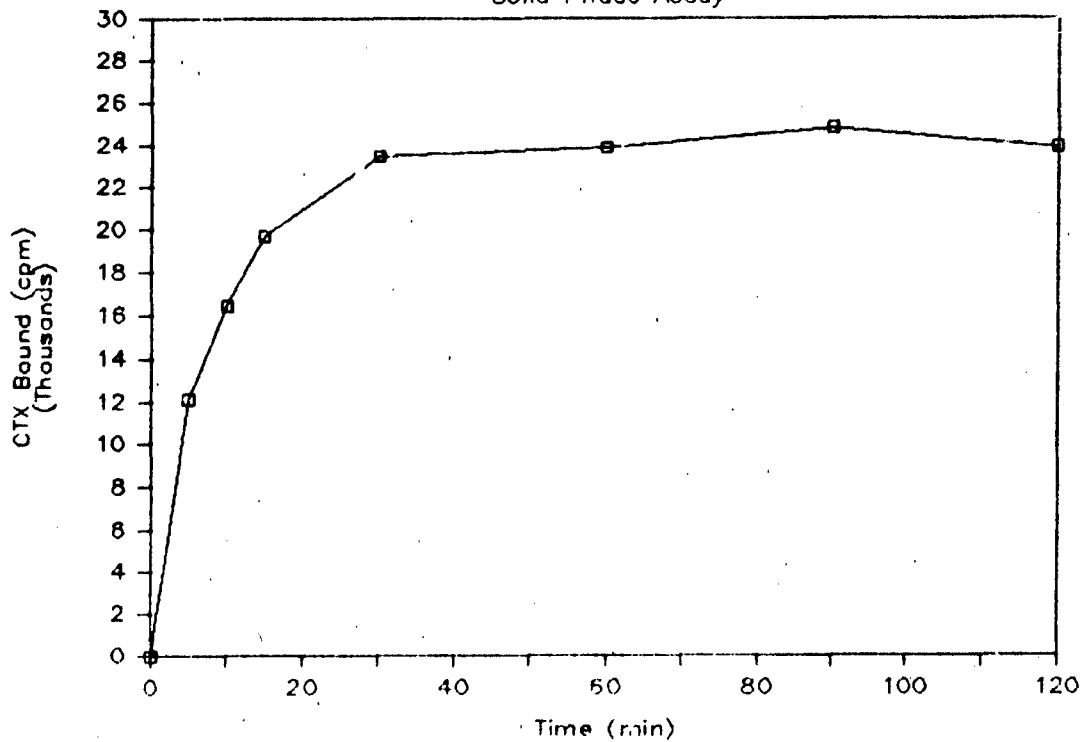


Fig. 10. ^{125}I -CTX association with AChR. Solid phase assay. Wells of microtiter plates were coated with 0.1 μg AChR in coating buffer by centrifuging plates at 1000 X g for 30 minutes. Wells were washed, bound radioactivity removed and counted in a gamma counter. Values are the mean of three replicates.

CTX-AChR DISSOCIATION

Solid Phase Assay

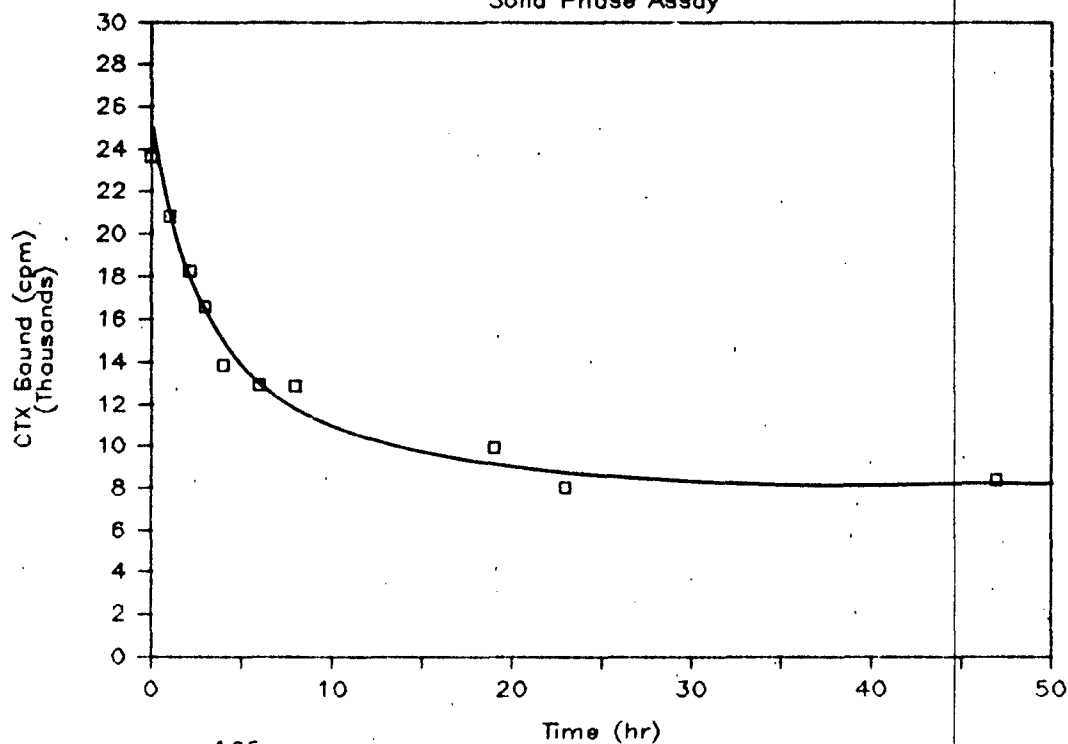


Fig. 11. ^{125}I -CTX-AChR dissociation. Solid phase assay. Wells of microtiter plates were coated with $0.25\text{ }\mu\text{g}$ AChR in coating buffer overnight at 4°C . Wells were washed, and quenched with 10% BSA for 30 minutes. ^{125}I -CTX ($6.9 \times 10^{-9}\text{ M}$) was added to wells and incubated for four hours. A 1000-fold excess of cold CTX was added to the wells. At increasing times, wells were washed and bound radioactivity removed. Values are the mean of three replicates.

CTX-AChR SATURATION

Solid Phase Assay

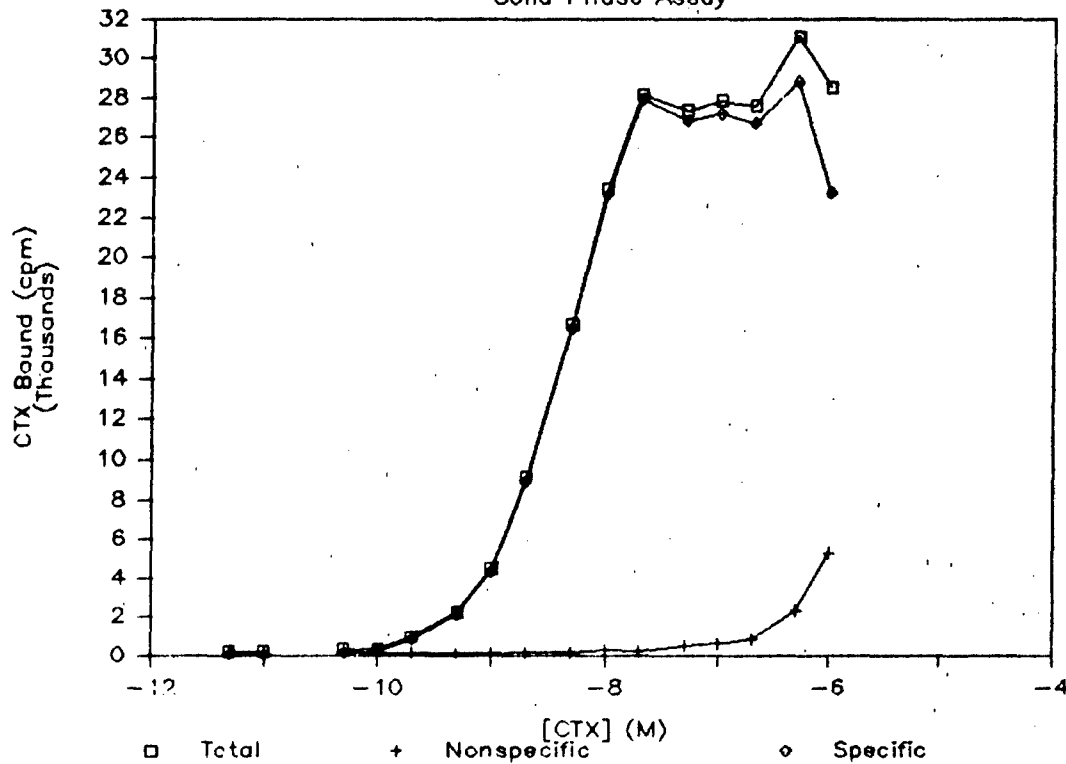


Fig. 12. Saturation of ^{125}I -CTX binding to AChR. Solid phase assay. Wells of microtiter plates were coated with 0.25 μg of AChR in coating buffer by centrifuging plates at 1000 X g for 30 minutes. Wells were washed, quenched with 10% BSA, and incubated with increasing concentrations of ^{125}I -CTX ($5 \times 10^{-12}\text{M}$ - 10^{-6}M) for 60 minutes. Bound radioactivity was removed and counted in a gamma counter to determine total binding. Non-specific binding represents binding observed in the presence of a 200-fold excess of cold toxin. This value was subtracted from total binding to determine specific binding. Values are the mean of four replicates.

CTX-AChR SCATCHARD PLOT

Solid Phase Assay

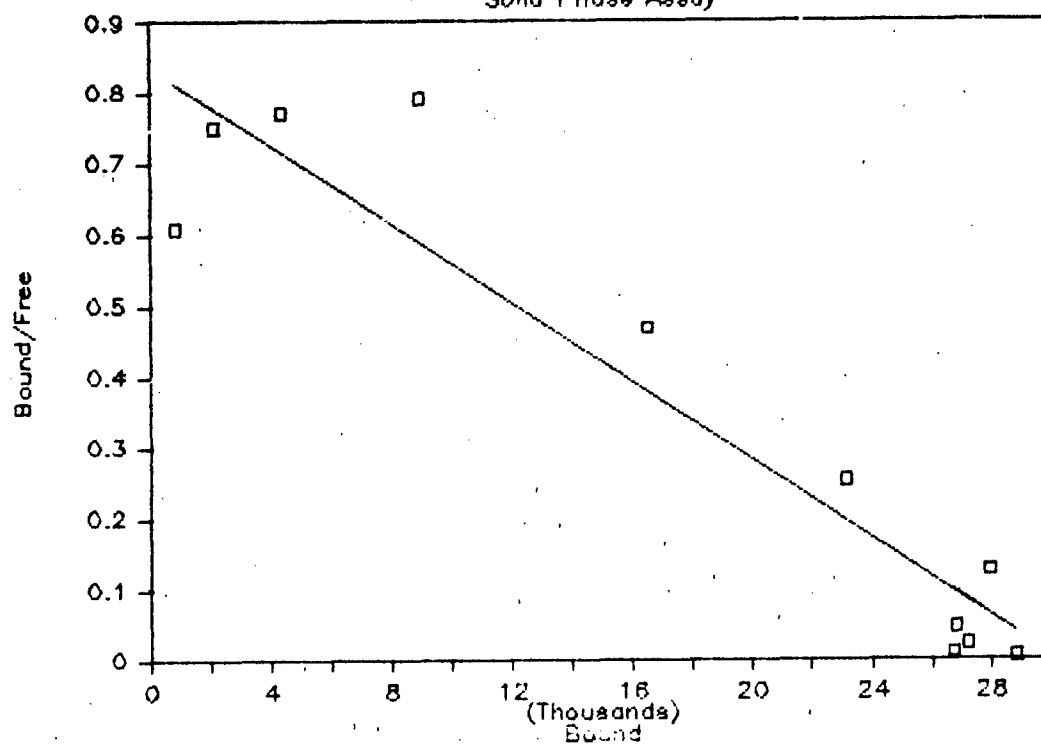


Fig. 13. Scatchard plot of equilibrium binding data in Fig. 12. Solid phase assay. Bound ^{125}I -CTX/free ^{125}I -CTX is plotted versus bound ^{125}I -CTX. The K_D calculated from the data is 2.7×10^{-9} M.

APAMIN-SYNAPTOSOME ASSOCIATION

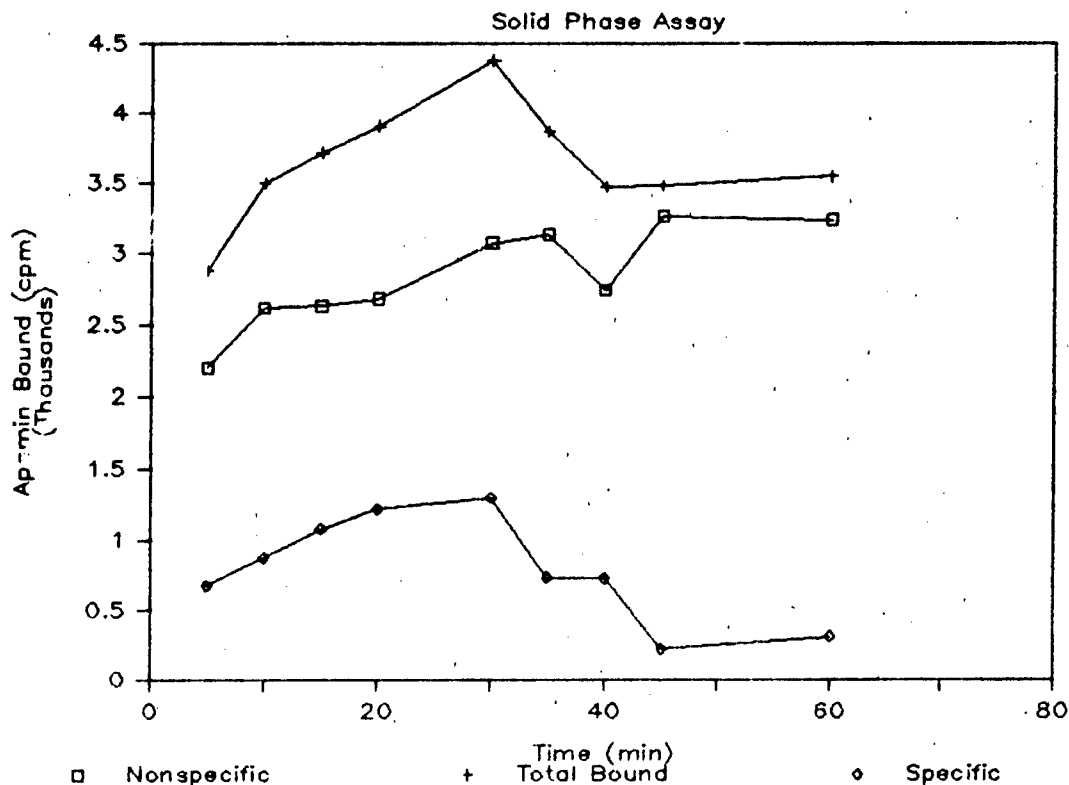


Fig. 14. Association of ^{125}I -apamin with synaptosome membranes. Solid phase assay. Wells of 24-well microtiter plates were coated with $250\text{ }\mu\text{g}$ membrane protein by centrifuging plates at $1000\times g$ for 30 minutes. Wells were washed, quenched with 10% BSA, and ^{125}I -apamin added (100,000 cpm). At increasing times, wells were washed and bound radioactivity removed. Values are the mean of four replicates.

Apamin-Synaptosome Saturation

Solid Phase Assay

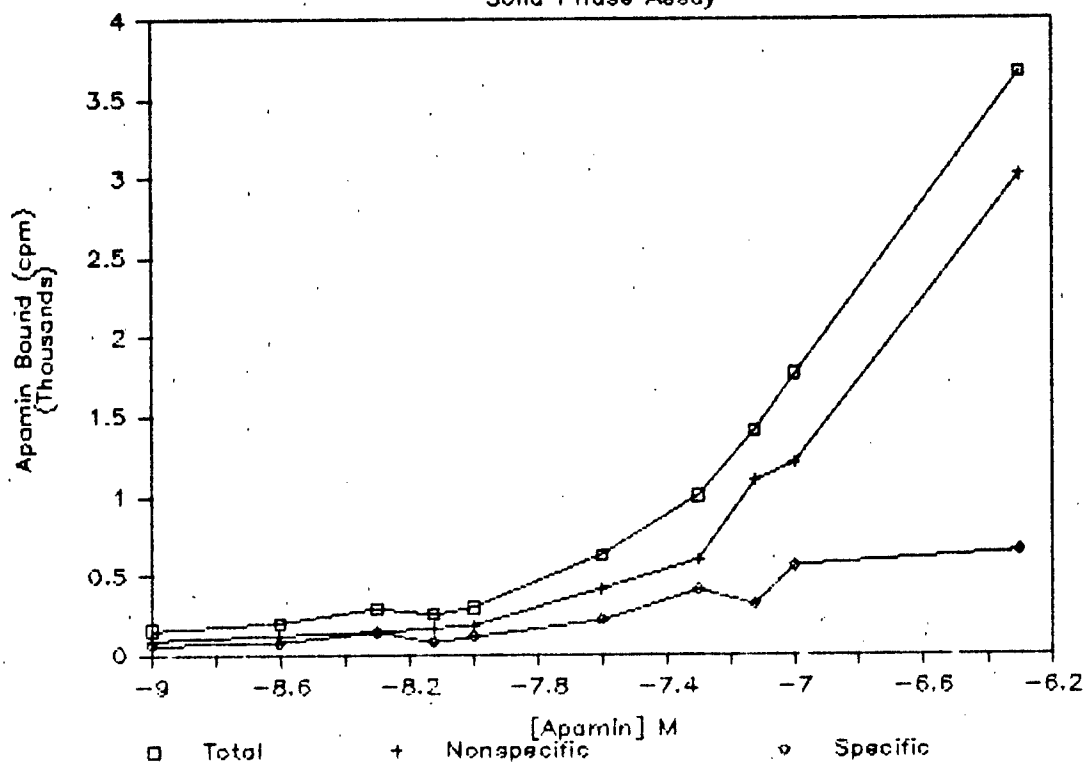


Fig. 15. Saturation of ^{125}I -apamin binding to synaptosomes. Solid phase assay. Wells were coated with 200 μg synaptosome membrane protein by centrifugation. After washing and quenching, increasing concentrations of ^{125}I -apamin were added and incubated for 30 minutes. Some wells were incubated in the presence of a 200-fold excess of cold apamin to determine non-specific binding. Bound radioactivity was removed and counted and specific binding determined by subtracting non-specific binding from total binding. Values are the mean of four replicates.

APAMIN-SYNAPTOSOME SCATCHARD

Solid Phase Assay

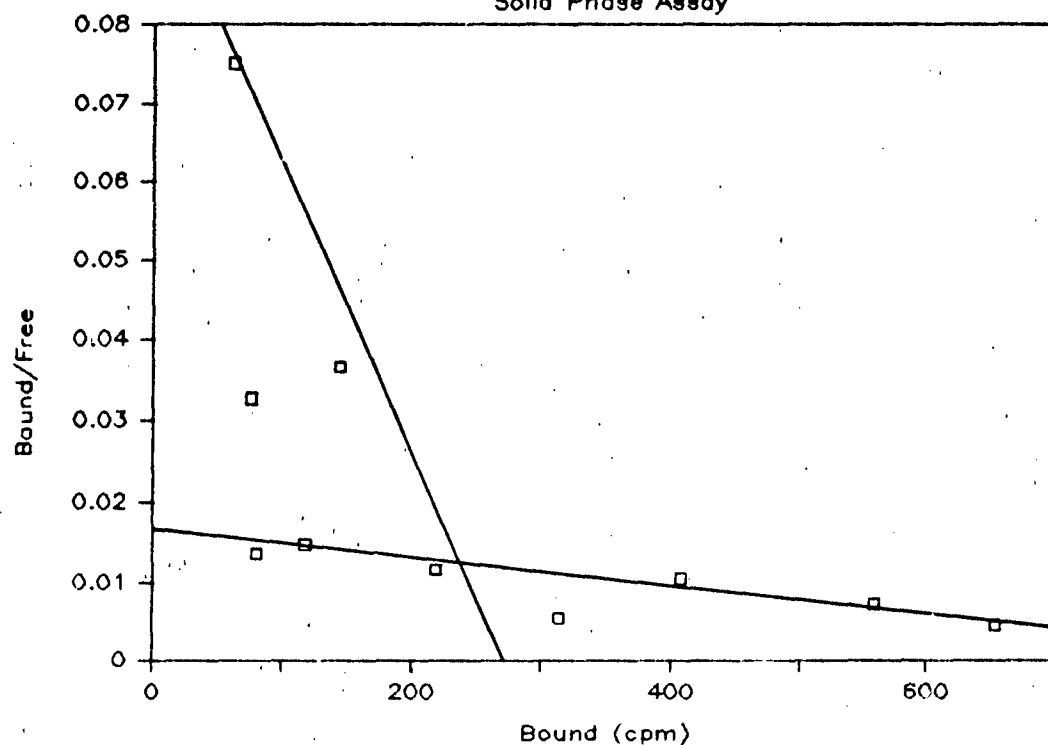


Fig. 16. Scatchard analysis of ^{125}I -apamin-synaptosome equilibrium binding data (Fig. 15). Bound apamin/free apamin is plotted versus bound apamin. A non-linear curve indicative of two binding sites is observed.

BTX-AChR ASSOCIATION

Filter Disk Assay

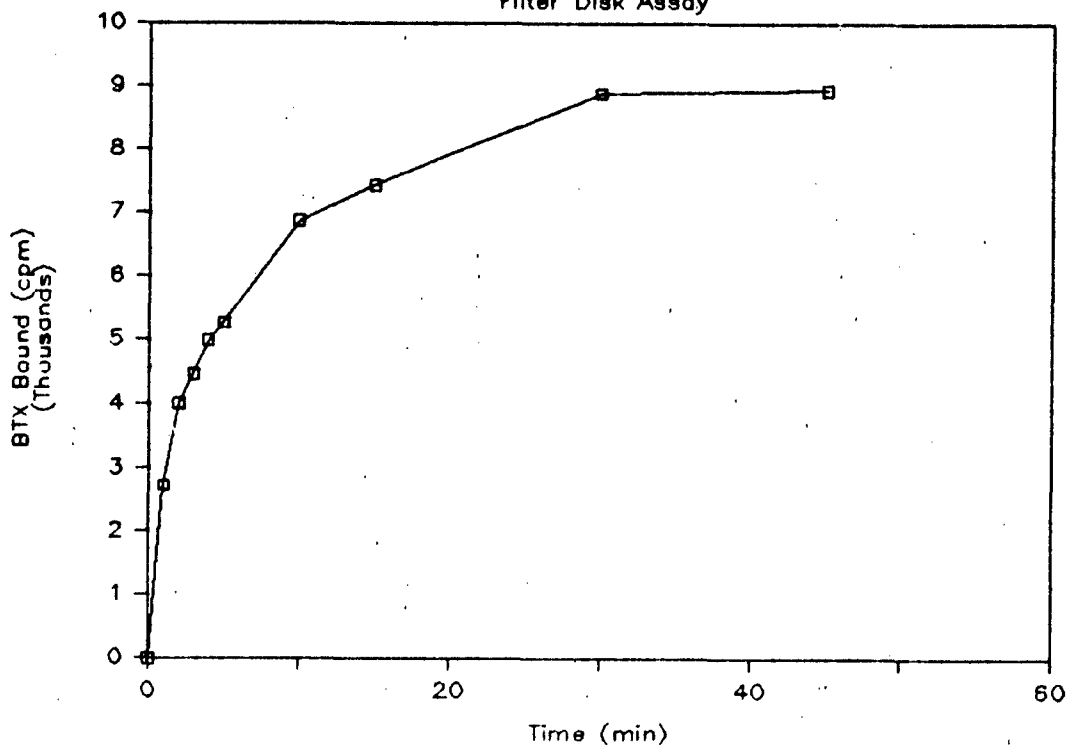


Fig. 17. ^{125}I -BTX association with AChR. Filter disk assay. ^{125}I -BTX (150,000 cpm) was incubated with AChR in tubes in 50 mM phosphate buffer, 0.1% Triton X-100, 0.005% NaN_3 , pH 6.5. A 1000-fold excess cold BTX was included in some tubes. Incubation was allowed to proceed for increasing times after which 50 μl of solution was spotted on a filter. Each aliquot contained 500 fmol AChR. Filters were washed three times and counted in a gamma counter. Radioactivity observed in the presence of excess cold toxin was subtracted from the radioactivity observed with hot toxin alone to determine specific binding. Values are the mean of three replicates.

BTX-AChR DISSOCIATION

Filter Disk Assay

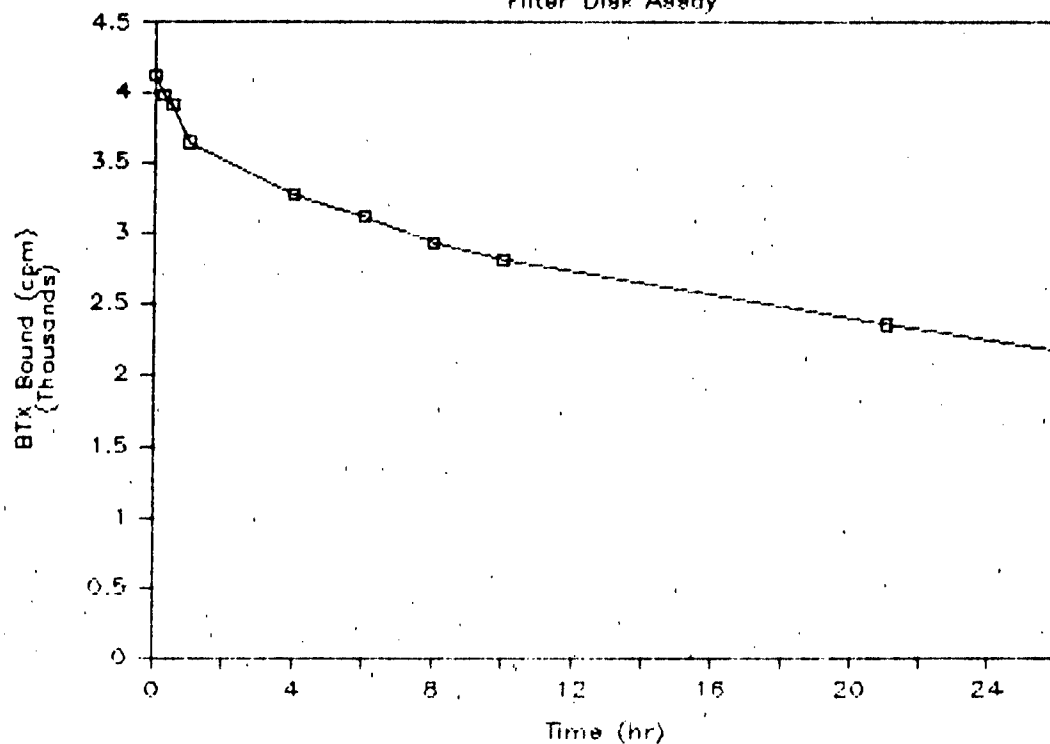


Fig. 18. Dissociation of ^{125}I -BTX from AChR. Filter disk assay. ^{125}I -BTX was incubated with AChR for 45 minutes after which 1000-fold excess cold BTX was added to the tube. At increasing time intervals, 50 μl of solution was spotted on a filter. The filters were washed and counted in a gamma counter. Background was the radioactivity bound after incubating hot and excess cold toxin for 45 minutes. Values are the mean of three replicates.

SATURATION OF BTX BINDING TO AChR

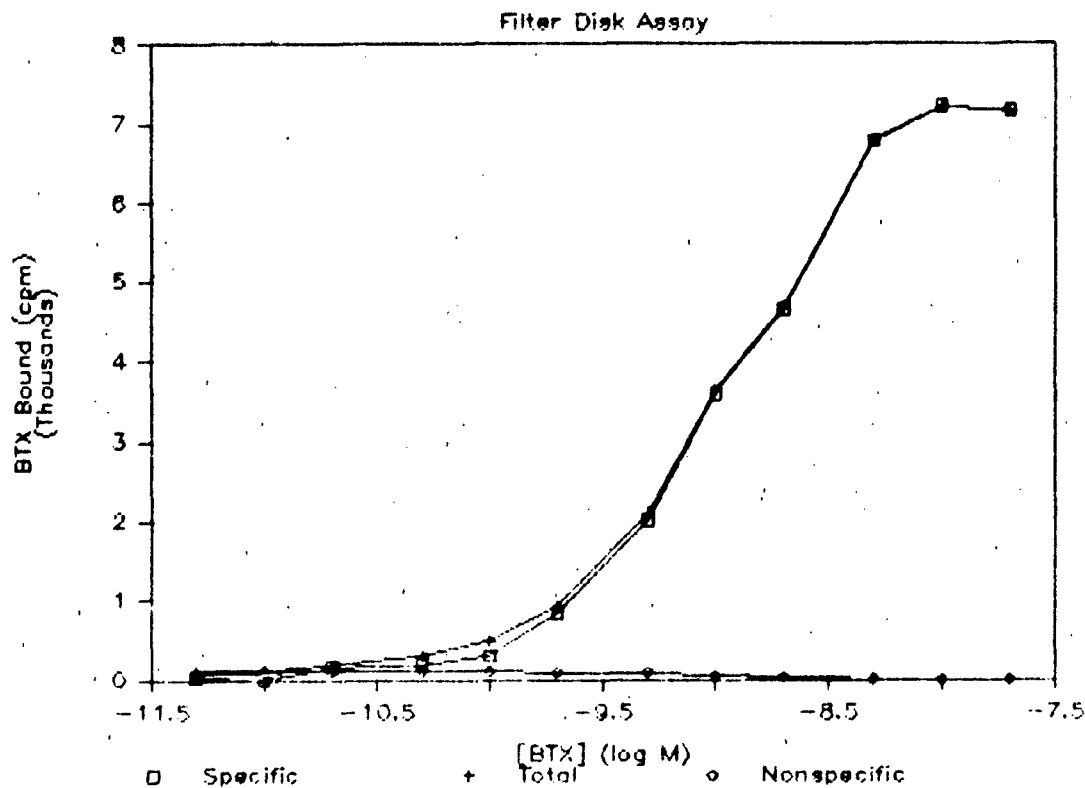


Fig. 19. Saturation of ^{125}I -BTX binding to AChR. Filter disk assay. Increasing concentrations of ^{125}I -BTX ($5 \times 10^{-12}\text{M}$ - $5 \times 10^{-8}\text{M}$) were incubated with AChR for 45 minutes. Fifty μl of solution were spotted on filters which were washed and counted in a gamma counter. Each aliquot contained 500 fmol AChR. Non-specific binding is the radioactivity bound in the presence of excess cold toxin minus the radioactivity bound after spotting hot toxin to the filters alone. Non-specific binding was subtracted from the total binding to determine specific binding. Values are the mean of three replicates.

SCATCHARD PLOT BTX-ACHR

Filter Disk Assay

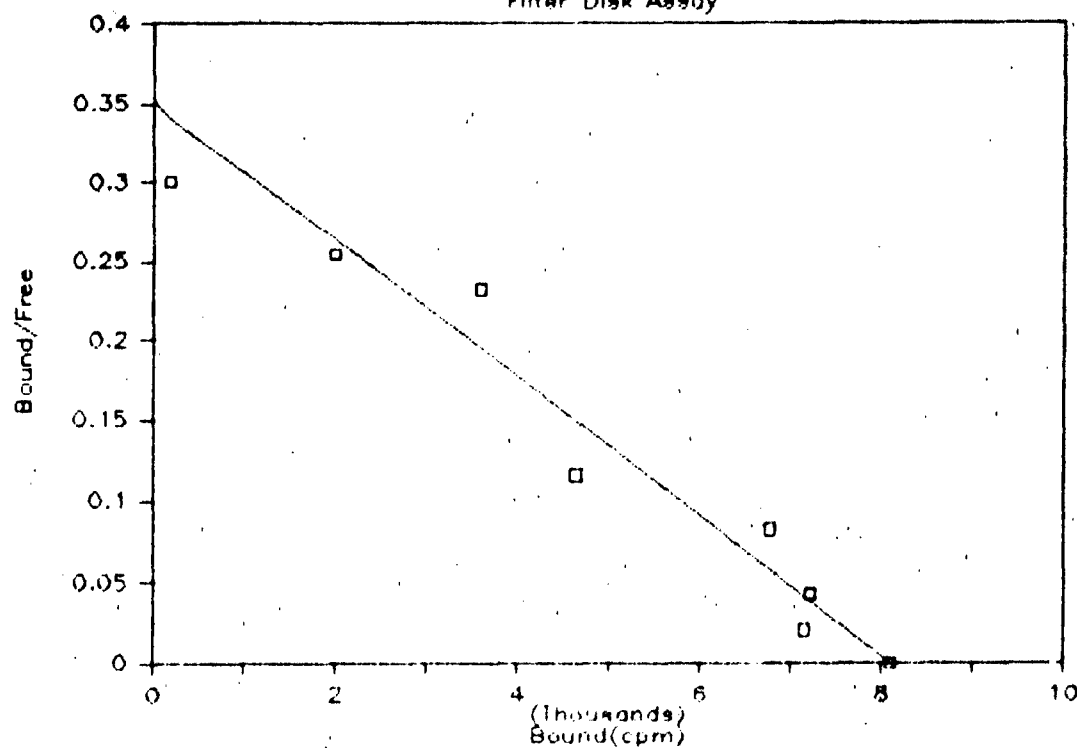


Fig. 20. Scatchard plot of equilibrium binding data in Fig. 19. Bound toxin/free toxin is plotted versus bound toxin. A linear plot is observed which yields a K_D of 2.8×10^{-10} M.

BTX-TORPEDO ASSOCIATION

Centrifugation Assay

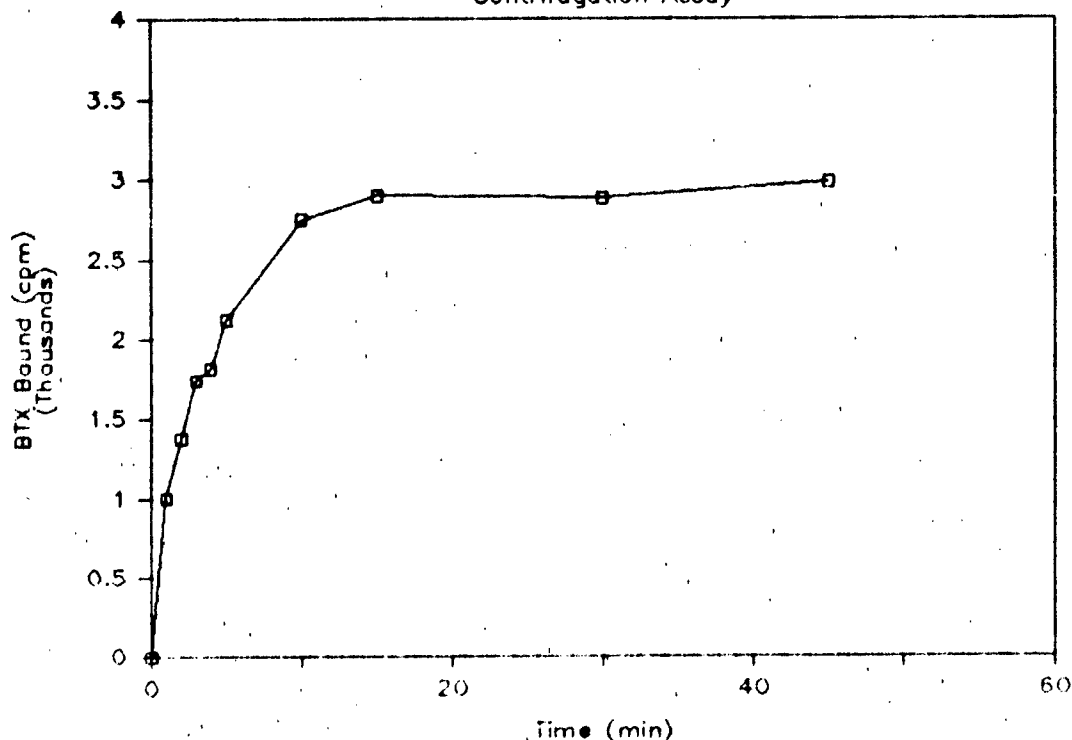


Fig. 21. Association of ^{125}I -BTX with AChR-rich Torpedo membranes. Centrifugation assay. Microcentrifugation tubes were quenched with 5% BSA. Ten μg of Torpedo membrane in 500 μl was added to tubes which were centrifuged for 10 minutes in a microfuge. The supernatant was aspirated and 500 μl of 1nM ^{125}I -BTX added to the tubes. At increasing time intervals, 1000-fold excess toxin was added to stop the reaction. The tubes were vortexed and centrifuged 10 minutes. The pellet was resuspended and washed three times with PBS. The final pellet was solubilized by SDS and counted in a gamma counter. Background binding observed in the presence of 1000-fold cold toxin was subtracted from experimental values. Values are the mean of three replicates.

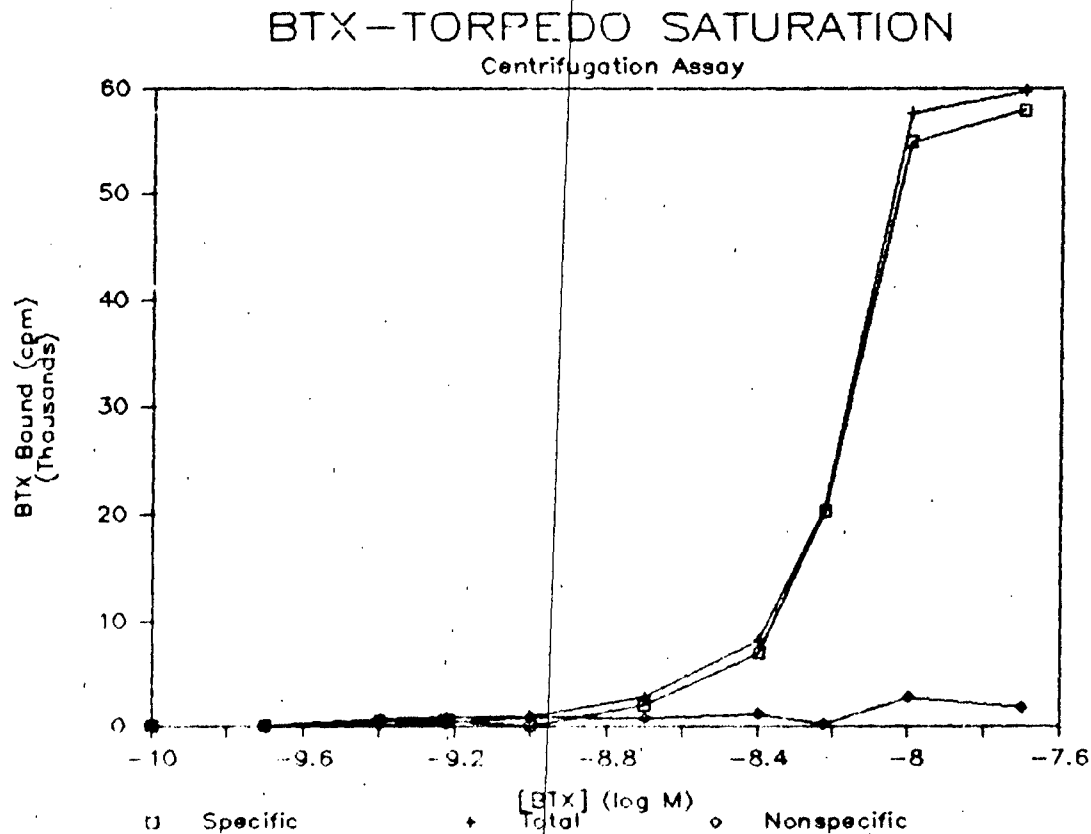


Fig. 22. Saturation of ^{125}I -BTX binding to Torpedo membrane protein. Centrifugation Assay. Labeled toxin in increasing concentration ($1 \times 10^{-10}\text{M}$ - $210 \times 10^{-8}\text{M}$) was incubated with $10 \mu\text{g}$ Torpedo membrane protein for 30 min, as described in Fig. 21. Background radioactivity measured in the presence of a 200-fold excess of cold toxin represents non-specific binding. This value was subtracted from total binding to obtain specific binding. Values are the mean of three replicates.

Binding inhibition assay. Experiments were performed to determine whether the solid phase assay could be used as a binding-inhibition assay that would be useful in screening potential inhibitors of toxin binding. The effect of cold BTX, α -cobratoxin, d-tubocurarine, nicotine, carbachol, choline, and sodium chloride on ^{125}I -BTX binding to purified AChR was tested (Figs. 23,24). BTX, α -cobratoxin, and d-tubocurarine are cholinergic antagonists, nicotine and carbachol are nicotinic agonists, and choline and sodium chloride are cationic agents. The concentrations of agents that produced 50% inhibition of binding (IC_{50}) of 5 mM ^{125}I -BTX are listed in Table 2.

Structure/Function Studies

The ability of a 32 amino acid peptide (32-mer) to bind ^{125}I -BTX in a solid phase assay was measured. This peptide comprises residues 173-204 of the Torpedo alpha subunit and contains cysteine-192 and -193. Peptide was adsorbed to the wells of 96-well polystyrene microtest plates as described for coating with AChR. Binding of labeled toxin to the adsorbed peptide was observed and could be competed by excess cold toxin (Fig. 25). To test the specificity of binding and obtain information on affinity, the ability of ligands to compete labeled toxin binding to peptide was measured. Employing the solid phase assay as a binding-inhibition assay, increasing amounts of ligand were incubated with 1 nM ^{125}I -BTX. The results of these experiments are illustrated in Fig. 25. The IC_{50} s of the agents tested are shown in Table 3. It was also possible to demonstrate binding by adsorbing toxin to the wells and incubating with iodinated 32-mer. Competition of labeled 32-mer by unlabeled 32-mer is illustrated in Fig. 26.

Preliminary experiments were undertaken to measure toxin binding to shorter peptides comprising portions of the 32-mer. These experiments yielded low levels of binding and high variability. Besides inability of peptides to bind toxin, these results could be due to inability of short peptides to adhere to plastic. Therefore, another assay which does not depend on immobilization of peptide was developed. In this assay, BTX is adsorbed to plastic and incubated with iodinated AChR. The ability of unlabeled AChR to compete with labeled AChR is shown in Fig. 27. It appears that this assay will be feasible for testing peptides, since, if they contain the binding site, they should compete with the labeled AChR for binding to the toxin.

Preliminary experiments were undertaken to compare the binding of ^{125}I -BTX to the Torpedo 32-residue peptide with corresponding peptides of calf and human α -subunit. In addition, toxin binding to a calf 32-mer in which glutamine was substituted for negatively-charged glutamate (position 180) was measured. Binding of 1 nM ^{125}I -BTX in the presence of 1 μM cold BTX is shown in Table 4. A greater reduction caused by cold toxin is indicative of a higher affinity of peptide for toxin. These results show that the Torpedo peptide binds toxin with higher affinity than the mammalian toxins and that the modification of glutamate 180 has little or no effect on binding.

Therapy

Production of antibodies against the 32-residue α -subunit peptide was undertaken as the first step in the long range goal of determining whether these might be effective in inhibiting toxin binding. Two rabbits were immunized with the 32-mer to raise polyclonal antibodies. The results obtained with one rabbit are shown in Fig. 28. This rabbit showed an increasing titer

BINDING-INHIBITION ASSAY

Inhibition of ^{125}I BTX Binding

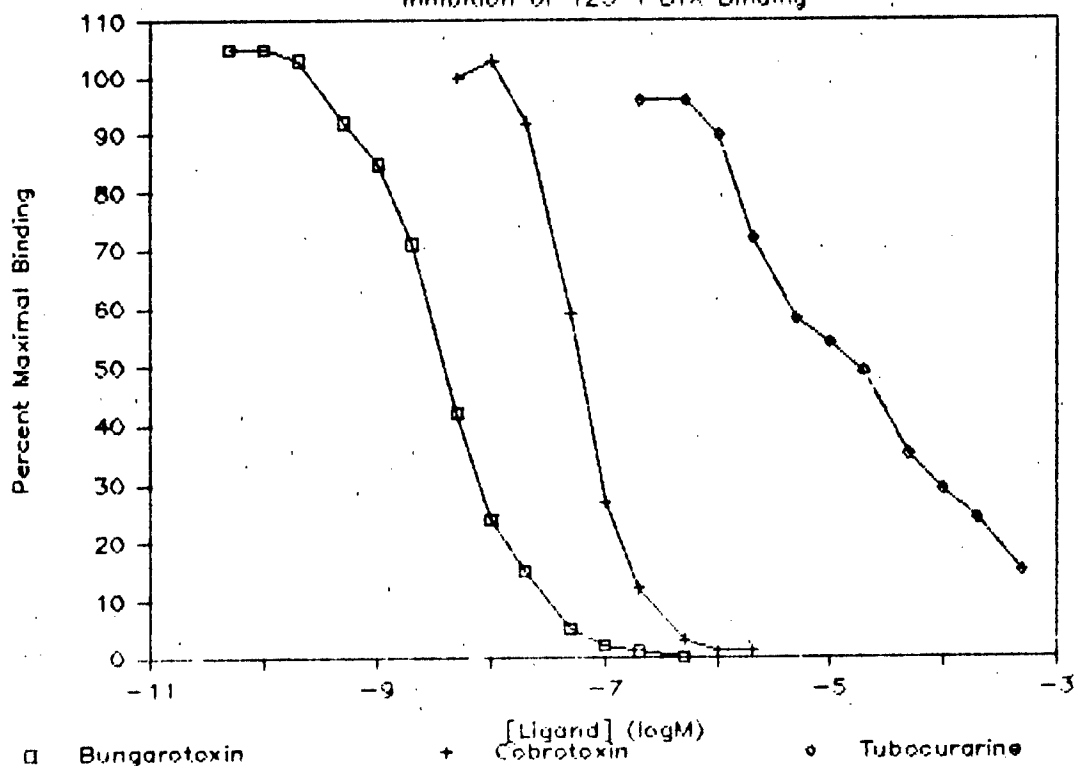


Fig. 23. Competition of ^{125}I -BTX binding to AChR. Solid phase assay. Wells of 96-well microtiter plates were coated with $0.15\ \mu\text{g}$ AChR and quenched with 5% BSA. After washing, $50\ \mu\text{l}$ of α -bungarotoxin, α -cobratoxin, or d-tubocurarine in increasing concentrations was added to the wells. Immediately thereafter, $50\ \mu\text{l}$ of 1nM ^{125}I -BTX was added to wells and incubated for 10 minutes. Wells were washed and bound radioactivity removed and counted in a gamma counter. Values are the mean of three replicates.

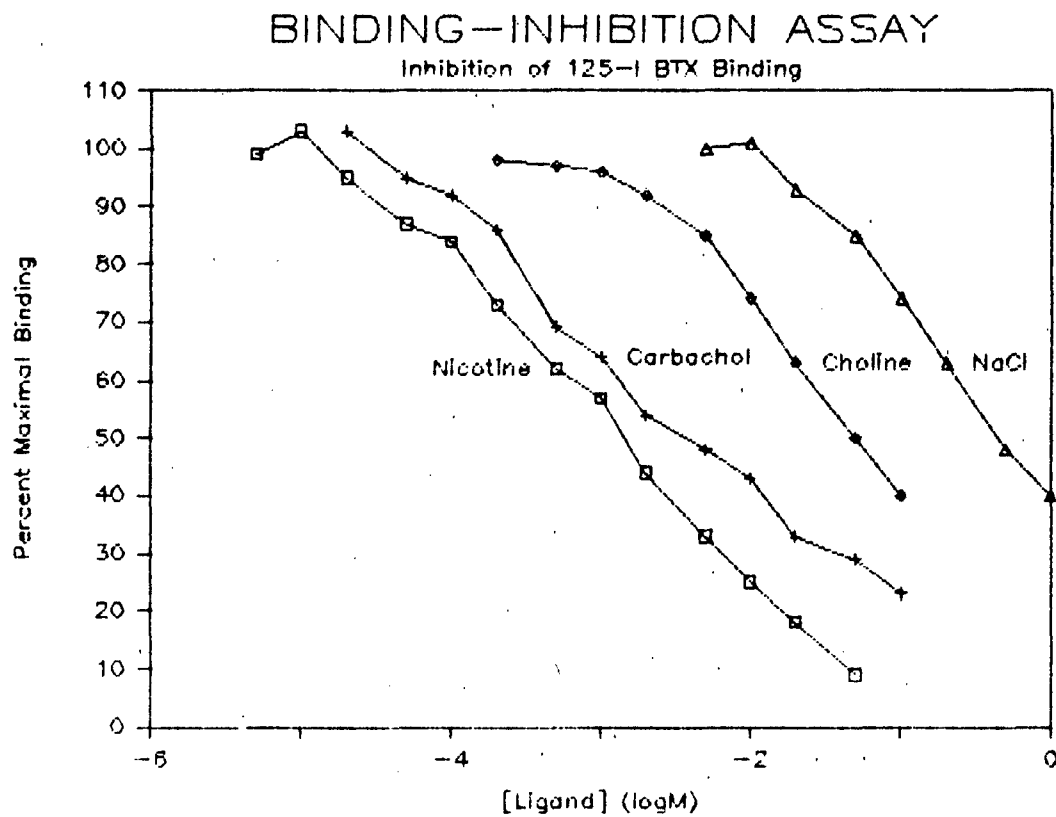


Fig. 24. Competition of ^{125}I -BTX to AChR. Solid phase assay. The effect of nicotine, carbachol, choline, and NaCl on toxin binding was measured as described in Fig. 23. Values are the mean of three replicates.

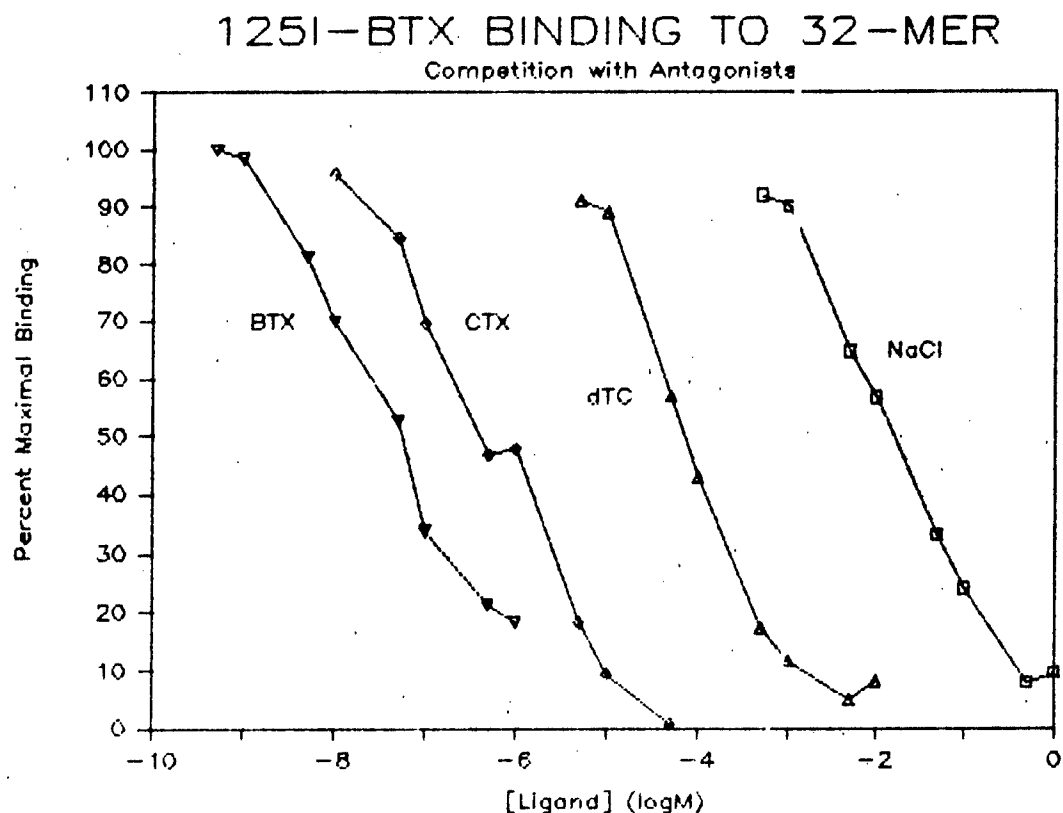


Fig. 25. Binding of ^{125}I -labeled BTX to Torpedo 32-mer and competition with antagonists. Wells of microtiter plates were coated with $5\text{ }\mu\text{g}$ 32-mer, quenched, and incubated with 1 nM ^{125}I -BTX. The effects of the following agents on toxin binding were tested: α -bungarotoxin (BTX), α -cobratoxin (CTX), d-tubocurarine (dTC), and NaCl. After washing, bound radioactivity was removed by SDS/NaOH and counted in a gamma counter.

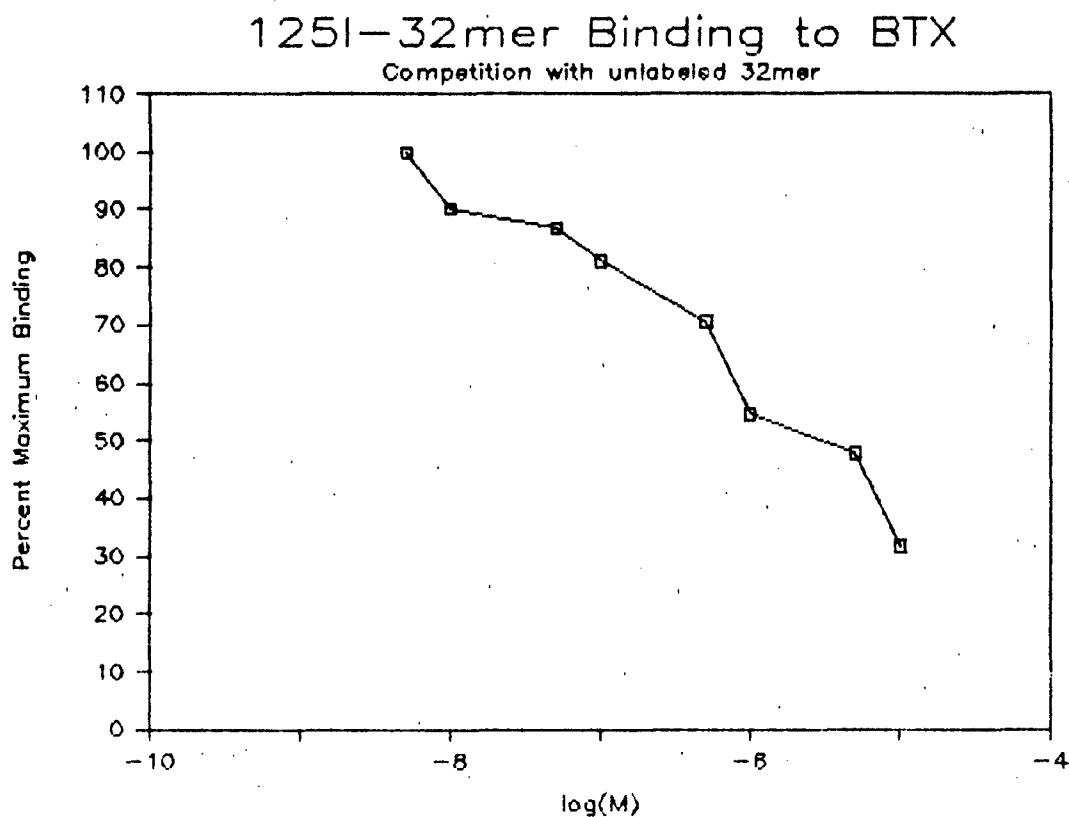


Fig. 26. Competition of ^{125}I -32-mer binding to BTX by cold 32-mer. Wells of microtiter plates were coated with $5\text{ }\mu\text{g}$ BTX, quenched, and incubated with ^{125}I -32-mer (100,000 cpm) in the presence of increasing concentrations of unlabeled 32-mer for 3 hours. Wells were washed and bound radioactivity removed and counted in a gamma counter. Values are the mean of three replicates.

125I-ACHR BINDING TO BTX

Competition with unlabeled AChR

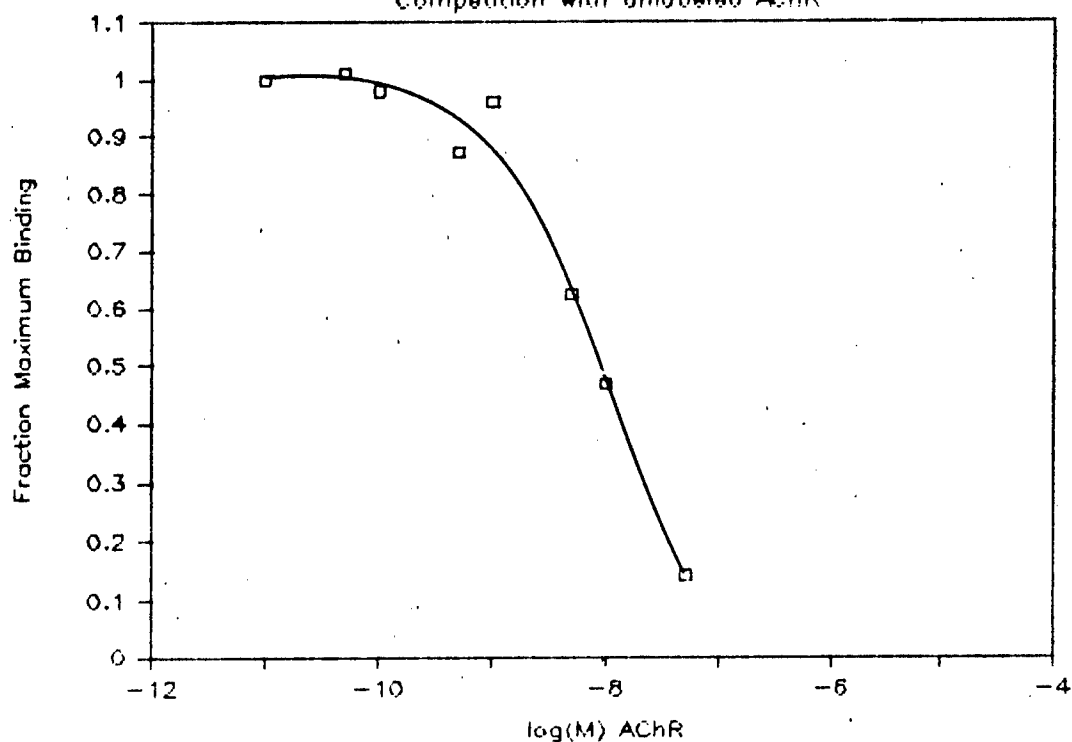


Fig. 27. Inhibition of ^{125}I -AChR binding to BTX by unlabeled AChR. Wells of microtiter plates were coated with 5 μg BTX. Wells were quenched with 2% BSA and incubated with ^{125}I -AChR (500,000 cpm) and increasing amounts of unlabeled AChR. After washing, bound radioactivity was removed by SDS and counted in a gamma counter.

Binding of anti-32mer antisera to 32mer

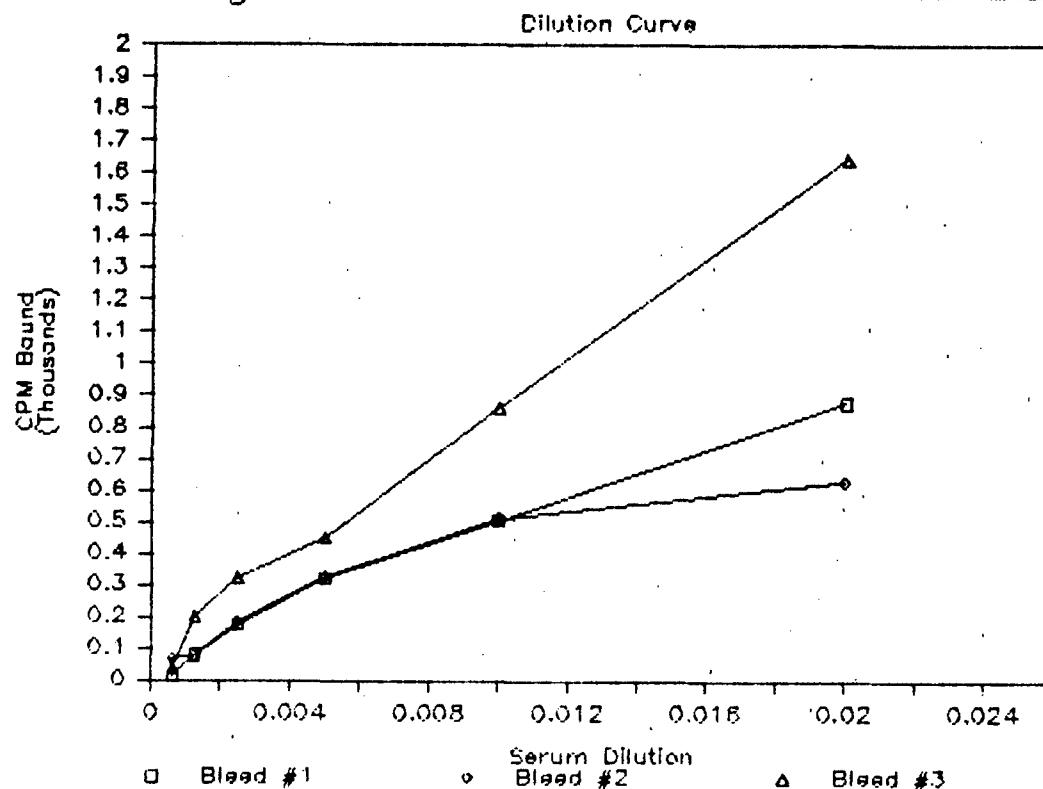


Fig. 28. Binding of anti-32-mer antisera to 32-mer. Antibodies were raised in rabbits against the 32-residue α -subunit peptide (32-mer) as described in Materials and Methods. To test reactivity of antisera, wells of microtiter plates were coated with 5 μ g of 32-mer. Wells were quenched and incubated with dilutions of sera for one hour. Wells were washed and incubated with 125 I-protein A (50,000 cpm) for one hour. Bound radioactivity was removed and counted in a gamma counter.

Table 2

Binding-Inhibition Assay, ^{125}I -BTX to AChR

<u>Agent</u>	<u>IC₅₀</u>
α -Bungarotoxin	$4.0 \times 10^{-9} \text{ M}$
α -Cobratoxin	$6.0 \times 10^{-8} \text{ M}$
d-Tubocurarine	$1.8 \times 10^{-5} \text{ M}$
Nicotine	$1.4 \times 10^{-3} \text{ M}$
Carbachol	$3.4 \times 10^{-3} \text{ M}$
Choline	$5.0 \times 10^{-2} \text{ M}$
NaCl	$> 1.0 \times 10^{-1} \text{ M}$

The concentrations of agents that produced 50% inhibition of 1 nM ^{125}I -BTX binding to AChR in the solid phase assay are listed. Based on experiments and data shown in Figures 23 and 24.

Table 3

Binding-Inhibition Assay, ^{125}I -BTX to 32-mer

<u>Agent</u>	<u>IC₅₀</u>
α -Bungarotoxin	$5.0 \times 10^{-8} \text{ M}$
α -Cobratoxin	$4.4 \times 10^{-7} \text{ M}$
d-Tubocurarine	$8.9 \times 10^{-5} \text{ M}$
NaCl	$1.6 \times 10^{-2} \text{ M}$

The concentrations of agents that produced 50% inhibition of 1 nM ^{125}I -BTX binding to 32 residue α -subunit peptide (32-mer) in the solid phase assay are listed. Based on experiment and data shown in Figure 25.

Table 4

 ^{125}I -BTX Binding to 32 Residue α -Subunit Peptides

<u>32-mer</u>	<u>% Residual Binding</u>
Torpedo	13.5
Human	66
Calf	40
Modified Calf	39

The reduction in binding of 1 nM ^{125}I -BTX to 32-mer by 1 μM unlabeled BTX was measured. Peptides (5 μg) were adsorbed to wells of microtiter plates. After quenching in 2% BSA, 1 nM ^{125}I -BTX and 1 μM cold toxin in 1% BSA in 10 mM phosphate buffer was added to the wells and incubated for 3 hours. Wells were washed and bound radioactivity removed and counted in a gamma counter. Values are the mean of three replicates.

against the 32-mer with successive boosts. The other rabbit showed a decrease in titer after the second boost. Antisera obtained after the first boost was tested for reactivity against the 32-mer, purified AChR, and AChR in receptor-rich Torpedo membranes (Table 5). Both antisera reacted with the 32-mer and receptor in Torpedo membranes. Only one antiserum, however, reacted with purified receptor. Binding of labeled BTX shows that the low binding to purified AChR is not due to lack of receptor.

In addition to polyclonal antibodies, monoclonal antibodies were raised in mice. Several clones producing antibody reacting against the 32-mer were obtained. Supernatants from the hybridoma cultures are being collected for concentration of antibodies. Further characterization of the antibodies has not yet been undertaken.

DISCUSSION AND CONCLUSIONS

A solid phase assay was developed to measure binding of ^{125}I -BTX to receptor-rich membranes from Torpedo electric organ and to AChR purified on a α -cobratoxin-Sepharose column. Comparable experiments were performed using two in vitro assays, a filter disk assay and a centrifugation assay. The affinity of toxin binding to the purified receptor was calculated from the rate constants determined from association and dissociation experiments and by Scatchard analysis of saturation data. The affinities obtained are shown in Table 6. In addition, the affinity of toxin binding to receptor-rich Torpedo membranes determined from a saturation experiment with the solid phase assay was 3.5×10^{-10} M. Both the rate of complex formation and dissociation were faster with the in vitro assay than for the solid phase assay. The dissociation constants determined from kinetic and equilibrium binding data were comparable, however. For both assays, the equilibrium K_D was one order of magnitude less than K_D from kinetic data. The rate constants observed with both assays fall within the range reported in the literature (15,47,51,52). The similarity in dissociation constants obtained with the solid phase, filter disk and centrifugation assays thus validates the solid phase assay as a useful technique for investigating toxin-receptor interactions.

Rate and dissociation constants were also determined for CTX (Naja naja atra), a short, postsynaptic neurotoxin (Table 6). With the solid phase assay, the rates of association and dissociation are faster for CTX than BTX, a long, postsynaptic neurotoxin. The dissociation constants determined from kinetic data and equilibrium binding are at least an order of magnitude lower than for BTX (Table 6). Chicheportiche et al. (13) reported that the dissociation constants for long and short neurotoxins are similar but that short toxins associate and dissociate from the receptor faster.

Efforts to measure binding of ^{125}I -labeled erabutoxin (ETX), another short neurotoxin, were unsuccessful. This may have been due to failure to adequately iodinate ETX. ETX has only one tyrosine residue at position 25, which may be buried in the molecule and not iodinated under mild conditions (see 53,47). CTX has an additional tyrosine at position 39 which is exposed. Thus, iodination of tyrosine 39 of CTX by the chloramine T method may explain why binding of this toxin could be demonstrated.

The solid phase assay offers several advantages over the in vitro assays. Because incubations are performed in the wells of 96-well microtiter plates, small amounts of materials and reagents (receptor and toxin) are required.

Table 5

Reactivity of Antisera to 32-mer with 32-mer and AChR

	<u>Serum No. 1</u>	<u>Serum No. 2</u> cpm bound	<u>Serum No. 3</u>
32-mer	878	971	---
AChR	111	1199	28814
<u>Torpedo</u> membranes	2848	1013	14249

The 32-residue peptide, purified AChR, and receptor-rich membranes from Torpedo electric organ were adsorbed to wells of microtiter plates. After quenching with 5% BSA, serum from rabbit number 1 and rabbit number 2 diluted 1:50 were added to wells. After washing, ^{125}I -labeled protein A was added, wells washed, and bound radioactivity removed and counted in a gamma counter. Wells containing AChR and membranes were also incubated with ^{125}I -BTX to obtain an estimate of the relative amount of receptor present.

Table 6

**Rate Constants and Dissociation Constants for ^{125}I -BTX
and ^{125}I -CTX Binding to Purified AChR**

Assay (Toxin)	K_{+1}	K_{-1}	K_d (Kinetic)	K_d (Equilibrium)
Solid Phase (BTX)	$3.4 \times 10^6 \text{ min}^{-1} \text{ M}^{-1}$	$1.5 \times 10^{-4} \text{ min}^{-1}$	$4.3 \times 10^{-11} \text{ M}$	$4.1 \times 10^{-10} \text{ M}$
Filter Disc (BTX)	$3.9 \times 10^7 \text{ min}^{-1} \text{ M}^{-1}$	$4.1 \times 10^{-4} \text{ min}^{-1}$	$1.0 \times 10^{-11} \text{ M}$	$2.8 \times 10^{-10} \text{ M}$
Solid Phase (CTX)	$1.2 \times 10^7 \text{ min}^{-1} \text{ M}^{-1}$	$1.8 \times 10^{-3} \text{ min}^{-1}$	$1.5 \times 10^{-10} \text{ M}$	$2.7 \times 10^{-9} \text{ M}$

Rate and dissociation constants for ^{125}I -BTX and ^{125}I -CTX to affinity purified Torpedo AChR were calculated from the data reported in the Results Section as described by Rylund (48).

Replicates are easily performed. Washing steps can be performed quickly using an aspirator and a multichannel pipette. Similarly, competition experiments are easily performed by adding inhibitors to the wells. Background binding (binding of labeled toxin in absence of receptor) is negligible in the solid phase assay. In the centrifugation assay, the signal/background ratio is about 10:1 and in the filter disk assay about 5:1. Background increases significantly in the filter disk and centrifugation assay at high toxin concentrations. Apparently, BTX binds less to the polystyrene plastic of microtiter plates than to DEAE-cellulose filter paper and polypropylene microcentrifugation tubes. Variability in experiments performed at different times and in replicate samples is lowest with the solid phase assay and highest with the centrifugation assay.

Some differences were noted between the results obtained with the solid phase and *in vitro* assays. The rate of association of toxin with the receptor was slower with the solid phase in comparison with the *in vitro* assay. Second, the affinities of agonists and antagonists of BTX binding as determined by IC_{50} values in the solid phase assay are at least one order of magnitude less than the affinities generally reported in the literature. The IC_{50} values observed with the solid phase assay are consistent, however, with the relative affinities of these agents for the AChR. Thus, the solid phase assay is most useful in comparing the relative effectiveness of agents that inhibit toxin binding.

Differences in the solid phase and *in vitro* assays may occur because in the former, toxin binds to receptor immobilized on plastic, while in the latter, it interacts with receptor in solution. In addition, in the filter disk assay, binding takes place in the presence of 0.1% Triton X-100. With the filter disk assay alone, differences in toxin binding to solubilized, purified and to the membrane-bound AChR have been observed (51). Solubilized receptor binds toxin more rapidly than membrane-bound receptor. Differences in rate constants could be due to different conformations for the soluble, purified and membrane-bound receptor, with toxin binding more rapidly to the solubilized conformation, or because the solubilized receptor is homogeneously distributed in the reaction volume, while the membrane-bound receptor is not (51). Such factors probably play a role in toxin binding to receptor immobilized on plastic, since different surfaces of the receptor may randomly adhere to plastic. In this case, the toxin-binding domain may vary in its accessibility to toxin in solution.

Binding of apamin to brain synaptosomes could be measured using the solid phase assay. It was necessary, however, to utilize 24-well polystyrene plates, the wells of which are larger than those of the 96-well plates. Apamin acts on the central nervous system by binding to receptors for vasoactive intestinal polypeptide (54). This receptor is present in considerably lower density than AChR in electric organ membrane. Thus, the solid phase assay can be used with preparations containing a low density of receptors by immobilizing a larger amount of membrane.

In order to identify the toxin-binding site on the AChR, ^{125}I -BTX binding to a 32-residue peptide (173-204) of the *Torpedo* α -subunit was investigated. Previously, binding of toxin to the 32-mer was demonstrated using a dot-blot assay (39). Here, binding of toxin to the 32-mer was observed using a solid phase assay in which peptide was immobilized on plastic. Specificity of binding was determined by the ability of excess peptide in solution and cholinergic antagonists to compete BTX binding. The concentration of BTX that

inhibited 50% of ^{125}I -BTX binding was 5.0×10^{-8} M and the concentration of d-tubocurarine was 8.9×10^{-5} M. Earlier, it was shown that the IC_{50} for inhibition of ^{125}I -BTX binding to the isolated α -subunit immobilized on filters was 1×10^{-7} M and 9×10^{-4} M for d-tubocurarine (18). Thus, the affinity of toxin binding to the 32-mer is as great as the affinity for the intact but denatured α -subunit. It is concluded that the 32-mer is a major determinant of the BTX-binding site in the receptor. However, the affinity of BTX for the 32-mer and the α -subunit is less than that for the intact receptor. Thus, it is likely that other nonadjacent sequences contribute to the overall high affinity of binding observed with intact AChR and perhaps also to the formation of the acetylcholine binding site.

It is generally assumed that the quaternary ammonium group of acetylcholine and the guanidinium group of arginine-37 of the neurotoxins interacts with an anionic subsite on the AChR. Of the four negatively charged residues in the 32-mer, the charge at residue 195 is not conserved in α -subunits from other species (55). The negatively charged residues at positions 175 and 200 are also present on at least two of the three other subunits constituting the *Torpedo californica* AChR (32). Thus, only the negative charge at residue 130 is unique to the α -subunit and, therefore, represents a likely candidate for anionic subsite.

To further localize the site, shorter peptides comprising portions of the 32-mer were synthesized. To date, binding of toxin to the shorter peptides has not been characterized because of difficulty in immobilizing short peptides. However, an assay in which receptor peptides are tested for ability to compete labeled intact receptor binding to BTX shows promise. Neumann et al. (40) reported binding of BTX to a 12-amino acid peptide comprising residues 185-196 of the α -subunit. However, the affinity of toxin binding to the 12-mer is 3.5×10^{-5} M (55), considerably less than the affinity we observe for BTX binding to the 32-mer (5.0×10^{-8} M). It does not seem likely that this short peptide contains all of the determinants for binding. To determine the importance of the negative subsite at position 180, a calf 32-mer was synthesized in which glutamine was substituted for glutamate-180. Little difference in binding of toxin to unmodified and substituted peptide was observed. This finding suggests that position 180 may not represent the negative subsite. Alternatively, since the toxin most likely interacts with multiple sites on the receptor, alteration of a single residue may not significantly affect overall binding.

Binding of labeled toxin to 32-residue peptides representing residues 173-204 of *Torpedo*, calf, and human α -subunits were compared. Surprisingly, affinity of toxin binding to the mammalian peptides was considerably less than to the *Torpedo* peptide. Similarly, Neumann et al. (56) reported that the human 12-mer (185-196) did not bind BTX in contrast to the *Torpedo* 12-mer. Most studies on toxin affinities have been performed using receptor derived from electric organ and we are unaware of any reports on toxin binding to the human receptor. It is possible, as suggested by these observations, that the human receptor does not bind BTX with as high affinity as *Torpedo* AChR. In this case, other toxins in the snake venoms may make a greater contribution to the overall toxic effects of the venoms on humans. This is an interesting question that deserves further investigation.

Both the calf and human peptides differ from the *Torpedo* 32-mer in that a serine is present at position 131 in the former in place of a tyrosine in *Torpedo*. Because aromatic-aromatic interaction is important for protein

structure stabilization (57), the lack of a tyrosine may indicate the human receptor has a different conformation than the Torpedo receptor, possibly accounting for the lower affinity of toxin binding.

Both polyclonal and monoclonal antibodies were raised against the Torpedo 32-mer. These results demonstrate that the peptide is sufficiently antigenic to elicit an immune response. The antibody titer in rabbits was relatively low, however, indicating that the hybridomas represent a better source of antibodies. Polyclonal antibodies were found to cross-react with purified AChR and membrane-bound AChR. This finding indicates the region of the receptor comprised by the 32-mer is accessible and not buried, as would be expected for the toxin binding site. One antiserum reacted with membrane-bound receptor but not isolated AChR. Such differences in reactivity are not uncommon and most likely are due to dependence of antibody binding on receptor conformation. The results of the antibody studies thus far demonstrate the feasibility of generating antibodies with binding-site peptides. Future studies can determine if these antibodies block toxin binding to the receptor.

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Lentz, T.L., P.T. Wilson and E. Hawrot. Binding of synthetic rabies virus glycoprotein peptides and α -bungarotoxin peptide with the acetylcholine receptor. *Neurosci. Abstr.* 12:236 (1986).

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Personnel Receiving Contract Support

Thomas L. Lentz, M.D., Professor of Cell Biology
Maria Gastka, Assistant in Research I
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